A STUDY OF EPSTEIN-BARR VIRUS (EBV)-TRANSFORMED CELLS *IN VITRO*.

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Abbreviations

Abbreviations which have been used in this thesis are as indicated in Biochemical Journal to authors which was revised in 1978 (Biochemical Journal 169, 1-17), with the following additions :-

ACIF	Anticomplementary immunofluorescence.
BL	Burkitt's lymphoma
B9	EBV B958
DNP	di-nitrophenol
EA	Early antigen
EBNA	Epstein-Barr Virus Nuclear Antigen
EBV	Epstein-Barr Virus
EM	Electron Microscopy
EMA	Early membrane antigen
FITC	Fluorescein isothiocyanate
Fc	Crystallizable fragment of Ig molecule
GAR	Goat anti-rabbit
GCs/C	Genome copies per cell
GN	Genome negative
GP	Genome positive
HUCL(s)	Human umbilical cord lymphocyte(s)
HPBL(s)	Human peripheral blood lymphocyte(s)
IF	Immunofluorescence
Ig	Immunoglobulin
IgG, IgM, IgA	Immunoglobulins G, M, and A classes
Ig(k), and Ig(λ)	Immunoglobulin k and λ subclasses
IM	Infectious mononucleosis

sIg, cIg	surface Immunoglobulin, cytoplasmic Immunoglobulin
LCL	Lymphoblastoid cell line
LMA	Late membrane antigen
NPC	Nasopharyngeal carcinoma
PAGE	Polyacrylamide gel-electrophoresis
PBS	Phosphate buffered saline
QW	EBV QIMR-WIL
RAH	Rabbit anti-human
RAM	Rabbit anti-mouse
Rb IgG	Rabbit Immunoglobulin G
TCA	Trichloroacetic acid.

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SUMMARY

The aim of my studies for this thesis was to establish and characterize pairs of EBV-transformed lymphoblastoid cell lines (LCLs) which resulted from the transformation of human umbilical cord blood lymphocyte (HUCL) populations, by different strains of EBV. EBV-transformed human LCLs have been shown to represent bone marrow (B)-derived cell populations. Moreover, the immunoglobulin (Ig) synthesizing cells *in vitro* among LCLs are thought to represent populations of lymphocytes which had been synthesizing Ig *in vivo* (Steel *et al.*, (1977)). LCLs are also thought to contain in their populations, other B-cell types. Finally, different strains of EBV are thought to have different biological characteristics *in vitro*. Two main premises to be tested were :-

(1) strains of EBV have different characteristics *in vitro*

and

(11) EBV transforms both Ig as well as non-Ig synthesizing cells, that is, individual LCLs should represent different populations of Ig and non-Ig synthesizing cells *in vivo*.

Each pair of cell lines was obtained from one common pool of HUCL population.

HUCL populations have been shown to be devoid of natural killer cells and EBV-specific cytotoxic thymus (T)-derived cells. This feature of HUCL populations, renders them excellent tools for the study of EBV-

induced transformation *in vitro*.

I established and characterized 16 pairs of LCLs. One of two pools from each of 16 samples of HUCL populations was either EBV (B958) or EBV (QIMR-WIL) transformed. Transformed cells were characterized after the establishment of the transformed state as judged by acidity in the growth medium, coupled with the appearance of transformed foci of cells.

The time lapse between infection of HUCLs and the establishment of the transformed state was shorter among EBV (B958) than among EBV (QIMR-WIL) transformed cells. The difference in the time lapse among the two groups of infected cells was attributable to differences in the transforming efficiences (TE). While EBV B958 had TE of $10^{3.4}$ transforming $dose_{50}$/ml (TD_{50}/ml), TE for EBV QIMR-WIL was found to be $10^{1.0}$ TD_{50}/ml.

The higher TE for EBV B958 was attributed to the characteristic features of the virus and the B958 cells. It was found that more than one EBV DNA core could become encapsidated in the EBV B958 capsid. This feature could contribute to the high transforming efficiency for EBV B958. B958 cells were found to survive for up to ten days in culture without medium replenishment, and a high viability among the cell populations retained. On electron micrographs, encapsidated EBV B958 particles were seen attached to the surfaces of undisrupted B958 cells. A cyclical phenomenon was proposed whereby, EBV B958

particles once released from disrupted cells, reinfect viable cells still in culture. The reinfected cells subsequently lysed and released viral particles, thus contributing to the maintenance of a high TE for EBV B958.

All 32 cell lines were found to synthesize the EBV-associated nuclear antigen, EBNA. There was no significant difference in the percentages of EBNA synthesizing cells between EBV (B958) and EBV (QIMR-WIL) transformed cells. However, EBV (B958) transformed cell lines were found to represent a higher number of producer cell lines than EBV (QIMR-WIL) transformed cell lines.

EBV (B958) transformed cell populations had higher percentages of Ig synthesizing cells than EBV (QIMR-WIL) transformed cell populations. EBV (B958) transformed cells had percentages of IgM and IgG synthesizing cells which were comparable to the percentages of IgM and IgG synthesizing cells among uninfected, untransformed HUCL populations. On the other hand, EBV (QIMR-WIL) transformed cell populations had fewer Ig synthesizing cells than uninfected, untransformed HUCL populations.

Among long-term cultures of both EBV (B958) and EBV (QIMR-WIL) transformed cultures, populations of cells were selected for continual proliferation, which had higher percentages of EBV-associated early antigen positive $(EA)^+$ cells, than newly established cultures. Among these $(EA)^+$ cell populations, there was also increased percentages of cytoplasmic Ig positive cells. It was therefore postulated that EBV-transformed cells

in culture could proliferate and synthesize Ig as a direct response to EBV-associated antigenic stimulation.

One factor was very obvious among long-term cultures which were analyzed by SDS-polyacrylamide gel-electrophoresis. Irrespective of the EBV strain which was used to induce transformation, the cells which survived in culture, were IgM synthesizing.

INTRODUCTION

ERRATUM

ECoR 1, read EcoR 1

1. EPSTEIN-BARR VIRUS (EBV)-ASSOCIATED DISEASES.

1.1. Burkitt's Lymphoma

In 1958, Dennis Burkitt found that a sarcoma of the jaws of African children was the most commonly occurring malignant tumour of African children who were attended to at the Mulago hospital in Uganda. He found that the tumour syndrome accounted for more than 50% of all children's cancers which were seen in Uganda, and the sarcoma was distinctly associated with the cells of the lymphoid system. Later in 1962, he found that the disease was not any more common in males than in females. Nearly all cases which were recorded occurred between the ages of 2 and 14 years, with a peak incidence at 5 years. The tumour syndrome was then referred to as Burkitt's lymphoma (BL).

One of Dennis Burkitt's colleagues, Professor Davis, who was based at the Makerere University in Uganda, suggested that the lymphoma could have been associated with a virus. They took biopsy cells from one such BL patient and dispatched these cells to M.A. Epstein *et al.*, (England) who investigated them by electron microscopy. M.A. Epstein and Yvonne Barr (1964) found that the BL cells contained numerous Herpes-type virus particles which they referred to as Epstein-Barr virus (EBV). The event established the association of BL with EBV.

In 1976, G. Bornkamm *et al.*, analyzed biopsy cells which had been taken from a European patient who had histologically typical BL, by nucleic acid hybridization with

EBV DNA. They found that the biopsy cells contained EBV DNA. Also in 1976, J.L. Ziegler et al., found that 20 American patients who had BL, had serological profiles which were very similar to those of African patients. BL can therefore occur as far afield as the United States, Europe, as well as Africa.

In 1970, P. Fialkow et al., looked at the genotype of normal and malignant tissue of seven patients who had BL and were heterozygous at the glucose-6-phosphate dehydrogenase locus. They found that while two enzyme types were observed in normal tissue, only one enzyme type was found in each of the tumour cells which had been examined. In one patient they found that four distinct tumours were found to have the same single cell type. This finding is indeed consistent with the hypothesis that individual BL tumours and perhaps the entire disease process has a clonal origin.

G. de Thé (1977) suggested that EBV could be reflective of an animal tumour virus whose oncogenic potential is enhanced by neonatal infection. R. Huebner et al., (1970) found that the C-type RNA tumour virus genome of mice, chickens, hamsters and cats was most commonly transmitted vertically as part of the genetic inheritance of most, if not all cells. They suggested that the group-specific antigen and the tumour were independently determined and could thus be switched on independently but were also observed together. There is no evidence which would implicate a genetic inheritance for EBV. However, the fact that the expression of BL is confined to infants in particular and mainly

juveniles in general, would endorse the suggestion of G. de Thé, that the development of EBV-oncogenesis could be associated with neonatal infection.

1.2. Infectious Mononucleosis

EBV is a ubiquitous virus as seen by its association with the internationally occurring, lymphoproliferative disease, infectious mononucleosis (IM). G. Henle et al., (1968) found that patients with IM regularly developed antibodies to EBV to acquire EBV seropositivity. The antibodies were distinct from the heterophile antibodies which are detectable in the acute phase of the disease. They stipulated that either EBV or a closely affiliated agent was responsible for the expression of IM. To date, no other agent has been discovered which satisfies the criterion as closely as EBV, consequently, it is generally accepted that EBV is the causative agent which stimulates the pathological expression of IM.

I. Royston et al., (1976) demonstrated that the peripheral blood lymphocytes (PBLs) which had been taken from IM patients were cytotoxic to bone marrow (B) derived but not to thymus (T) derived lymphoblastoid cell lines (LCLs). They suggested that the usual self-limiting course of IM may relate to the immunological control mechanisms which normally regulate the proliferation of B-cells in vivo. D.J. Moss et al., (1978) obtained PBLs from donors who had developed antibodies to EBV, seropositive donors, exposed the cells to EBV and subsequently cultured the infected cells

at various densities. They found that cells which had been cultured at very high densities produced transformed foci of cells within the first two weeks of culture but thereafter regressed. Subcultures of cells which were made four weeks after infection, rarely, if ever, gave rise to LCLs. A.B. Rickenson et al., (1980) investigated the development of EBV seropositivity among IM patients by the regression phenomenon. They found that there was development of a significant EBV-specific memory T-cell pool. EBV-specific memory T-cell activity was absent in the acute phase of IM, became evident at low levels 5-23 weeks after the onset of the disease and reached a maximum level after about 6 months. The development of EBV seropositivity in IM and among other seropositive donors is therefore dependent on the existence of a significant pool of EBV-specific memory T-cells.

1.3. Nasopharyngeal Carcinoma

Nasopharyngeal carcinoma (NPC) occurs in a markedly clustered geographical area. It is prevalent in Southern China, the adjacent Malaysian Archipelago, in a few small clusters of East Africa, Tunisia, and in some additional parts of the world. It is commonly found in adults between the ages of 40 and 50 years old in an approximate ratio of 2:1, males to females.

In animal species, the genes coding for molecules bearing various alloantigens which lead to rapid tissue graft rejection are located in the Major Histocompatibility Complex. In man, these genes coding for the alloantigens

are referred to as HLA and the genetic loci which are associated with their syntheses are : HLA-A, HLA-B, HLA-C and HLA-D. M.J. Simons et al., (1974) compared HLA profiles of predominantly Chinese patients with NPC, to those of suitably chosen controls. They found that there was a statistically significant elevation of the HLA haplotype, HLA-Az, among NPC patients.

To date, no data has been collated which would implicate a significant immunodef iciency or a genetic predisposition to EBV among BL and IM patients. On the other hand, among NPC patients there is a statistically significant probability that there may be a genetic predisposition to EBV-induced oncogenesis.

2. THE DEVELOPMENT OF EBV-INDUCED ONCOGENESIS IN VITRO.

2.1. Early Experiments On EBV-Induced Transformation in vitro

During the earlier experiments on transformation of cells by EBV in vitro, G. Henle et al., (1967) lethally X-irradiated cells from two BL-derived LCLs, Jijoye and Raji. They then co-cultivated the X-irradiated cells with PBLs from a female infant. They found that the PBLs had been induced to proliferate within 2-4 weeks when co-cultured with Jijoye but not when co-cultured with Raji cells. They suggested that, present among the populations of Jijoye cells were EBV-carrying cells which could induce the transformation and subsequent continual proliferation of EBV-susceptible cells in vitro. Moreover, if either PBLs or the X-irradiated cells were cultured alone the

cells did not survive for any length of time. To support their claim to fame, they found that among 100-300 cells which were in the metaphase stage of chromosomal replication, none contained a Y-chromosome. Since Jijoye had been derived from a male patient, they concluded that the LCL had indeed been established from the PBLs of the female infant.

The discovery of G. Henle et al., (1967) strongly points to the fact that, while some EBV-transformed LCLs carry infectious viral particles, others do not bear this characteristic. Since those early teething days of 1967, events have traversed a very long way in favour of the development of EBV-induced transformation in vitro. Transformation in this context is defined as :-

The morphological change in a proportion of the infected cells.

The event is associated with continual proliferation of the transformed cells, leading to the establishment of an EBV-transformed LCL.

2.2. Establishment of EBV-Transformed LCLs

Lymphoblastoid cell lines can be established from the tumour cells of NPC and BL patients as well as from the peripheral blood lymphocyte populations of IM patients. LCLs can also be established from subjects with unrelated (in respect of the accepted EBV-associated) diseases. M.A. Epstein et al., (1965) established BL-derived LCLs from the biopsy cells of BL patients. J. Pagano et al.,

(1976) successfully established EBV-infective LCLs from the PBLs of IM patients. In 1968, J.H. Pope established a LCL, QIMR-WIL, from the PBLs of a patient with myeloblastic leukaemia.

2.3. Time Lapse Between Infection and Proliferation

The cultural environment does govern the successful establishment of EBV-transformed LCLs. J.H. Pope (1968), and J.H. Pope et al., (1974) found that the time lapse between infection of cells and the outgrowth of EBV-transformed foci of cells which subsequently led to the establishment of LCLs, varied between 5 and 35 days post infection. T. Katsuki et al., (1975) found that the time lapse which varied between 7 and 15.7 days was directly related to the titre of infectivity.

2.4. Classification of Lymphoblastoid Cell Lines

EBV-infected LCLs had been classified by G. Klein et al., (1972) into two groups :-

1. Producers
2. Non-producers

These classifications have now been replaced by three terms :-

1. Restringent
2. Abortive
3. Productive

E. Kieff et al., (1979) have described these terms as :-

1. Restringently infected LCLs are cells which express the EBV-associated Nuclear Antigen (EBNA) and have an enhanced potential for continual proliferation *in vitro*.
2. Abortively infected LCLs are restringently infected cells which have been induced to synthesize the EBV-associated antigens; membrane antigen, MA, and early antigen, EA. The state of abortive infection can be brought about by the treatment of restringently infected cells with inhibitors of the cell cycle or by superinfection with extracellular virus. Abortively infected cells synthesize in addition to EBNA, MA, and EA and are associated with cellular RNA, DNA, and protein syntheses.
3. Productively infected LCLs (producers) express the additional EBV-determined virus capsid antigen, VCA.

The consequence of VCA production among a certain percentage of the producers, is the ultimate synthesis and/or release of mature and/or infectious viral particles.

3. SYNTHESIS AND RELEASE OF VIRAL PARTICLES

G. Klein et al., (1972) found that, among productively infected cells, only approximately 5% of the cells in the population spontaneously entered the productive cycle. About 2.5% of the cells which were found to be synthesizing the "early" products proceeded to the synthesis of the "late" products of the viral cycle, and < 2.5% released mature viral particles.

H. zur Hausen et al., (1967), looked at the fate of ^{3}H-Thymidine (^{3}H-TdR) in productively and restringently infected EB-3 and Raji cells respectively. Shortly after a two hour pulse, ^{3}H-TdR label as detected by autoradiography, could be seen in the nuclei of most EB-3 and Raji cells. In a small fraction (approximately 5%) of EB-3 cells, ^{3}H-TdR label displayed a focal distribution which was sometimes detectable in the cytoplasms within eight hours after the pulse. They compared their autoradiograms with EBV-specific immunofluorescence (IF), using human sera which contained antibodies to EBV-associated antigens. They found that, most of the IF-positive cells displayed focal labelling when they were observed at 24 and 48 hours. On the other hand, fewer cells were IF-positive and displayed focal labelling when they were observed at 8 and 72 hours.

H. zur Hausen et al., (1967), suggested that there was a constant turnover of cells among producers, which synthesize products which are associated with the EB viral cycle. They found that all the individual fluorescent

cells which had been picked, sectioned and observed by electron microscopy, contained numerous viral particles. This finding led these workers to suggest that the cells which were synthesizing virally induced products were also synthesizing viral particles. The time between infection of cells and the subsequent release of EBV particles was approximated to be four days.

4. EPSTEIN-BARR VIRUS STRAINS

J.H. Pope (1968) found that QIMR-WIL was productive in favour of the release of infectious viral particles. The virus, EBV QIMR-WIL, has been used extensively by many workers including D.J. Moss et al., (1972) and T. Katsuki et al., (1975) in their quest to elucidate EBV oncogenesis. Infectious virus has been recovered from the throat washings and epithelial tumour cells from NPC patients (C. Desgranges et al., 1977 and P.A. Trumper et al., 1977).

G. Miller et al., (1973) obtained a transforming strain of EBV from the IM-derived LCL, 883-L. They passaged EBV 883-L in marmoset cells (B958) and obtained a transforming strain of EBV, EBV B958, which had a greatly increased titre when compared with EBV 883-L. They suggested that B958 could have facilitated the envelopment of a greater number of EBV particles than 883-L cells. The cultural conditions of producer cells does influence the acquisition of infectious virus particles.

It is very debatable whether or not EBV P_3HR-1 does harbour transforming potential. T. Aya et al., (1974)

reported on the successful transformation of human umbilical cord blood lymphocytes (HUCLs) after infection with EBV P_3HR-1. J. Yata et al., (1975) suggested that the former workers could have used an "early" passage of the virus strain. EBV P_3HR-1 was derived from Jijoye cells as a subclone (G. Klein et al., 1979). W. Henle et al., (1967) successfully transformed infant's PBLs after infection with X-irradiated Jijoye cells. U. Schneider et al., (1975) described the transforming efficiency (TE) of a virus strain as the $\log_{10}$ of the virus dilution which induced 50% of replicate cultures (TD_{50}/ml) to proliferate (i.e. TE = TD_{50}/ml). T. Sairenj et al., (1980), recently re-evaluated the transforming potential of EBV Jijoye. They found that Jijoye when grown at a "shift-down" temperature of 33°C, released infectious virus particles and TE was found to be $10^{5.2}$ TD_{50}/ml.

5. WHICH CELLS ARE SUSCEPTIBLE TO EBV-INDUCED TRANSFORMATION IN VITRO ?

The question arises as to which cells are susceptible to EBV-induced transformation. The findings of P.A. Trumper et al., (1977) does implicate the susceptibility of the epithelial cells of NPC patients to EBV-induced transformation. However, the findings of M.J. Simons et al., (1974) would suggest that the predisposition of NPC patients to EBV oncogenesis could be genetically determined in vivo.

5.1. Surface Immunoglobulin Positive Cells and EBV Induced Transformation.

M. Jondal et al., (1973) obtained HUCLs, HPBLs, and EBV-transformed LCLs and exposed these cell populations to EBV receptor-positive cells, P_3HR-1. They found that; HPBLs, HUCLs, and LCLs formed rosettes with P_3HR-1 cells; suggesting that the lymphocyte populations bore EBV receptors. Moreover, when the cell populations were exposed to anti-immunoglobulin columns, they lost their capacity to form rosettes with P_3HR-1 cells. The lymphocyte populations were also shown to bear receptors for complement and the crystallizable fragment (Fc) of immunoglobulin molecules. They suggested that surface immunoglobulin positive (sIg^+Fc^+) B-cells, were indeed carriers of EBV receptors.

T. Katsuki et al., (1977) separated HUCL populations into subpopulations of sIg^+Fc^+ (B)-cells, sIg^-Fc^+ (Null) cells, and T-cells, and exposed the different subpopulations of cells to EBV. They found that while T-cells and Null-cells were unsusceptible, sIg^+Fc^+ (B)-cells were susceptible to EBV-induced transformation. Furthermore, the frequency of transformable cells correlated with the fraction of sIg(M)-bearing cells. They suggested that sIg(M)-bearing cells was the predominant subpopulation among HUCL populations which was susceptible to EBV-induced transformation in vitro.

M. Steinitz et al., (1980) preselected sIg(A)-positive cells from the peripheral blood of human subjects and

cervical lymph node lymphocytes of NPC patients. They successfully EBV-transformed the preselected lymphocytes. T. Katsuki et al., (1975) exposed HUCL, to EBV B958 and EBV QIMR-WIL to obtain LCLs. Within five months of establishment, they subjected the LCLs to direct IF. They found that the cell populations were sIgM-, and cytoplasmic (c)-IgG positive.

Thus, it does seem that, while sIg(M)-bearing cells might be the predominant cell type among HUCLs which are susceptible, other sIg^+ cell types are susceptible to EBV-induced transformation in vitro.

5.2. Macrophages and EBV-Induced Transformation

J.H. Pope et al., (1974) found that transformed foci of proliferating cells appeared in cultures of non-adherent cell populations from HPBLs when co-cultured with EBV QIMR-WIL, within 10-17 days of infection. When similar non-adherent cell densities were exposed to similar numbers of EBV QIMR-WIL particles in the presence of adherent cells, transformed foci appeared within 10 days of cultivation. The adherent cells absorbed Indian ink, were sIg^+ and had the morphological appearance of macrophages. They found that the non-adherent cells never developed transformed foci when they were freed of other cells and co-cultivated with EBV QIMR-WIL in vitro.

U. Schneider et al., (1975) used TE to investigate the phenomenon of macrophage enhancement of EBV-induced transformation of HUCLs. They found that TE was highest in

unfractionated lymphocyte populations, reduced after macrophage depletion, and was reduced even further after B-cell depletion. When the different subpopulations were reconstituted, TE optimal was not attained in cultures with a high density of B-cells, but in cultures with a high density of macrophages. They suggested that there was a co-operative effect expressed when B-cells were co-cultured with macrophages and macrophages enhanced the proliferation of EBV-infected HUCLs *in vitro*.

5.3. Human Fibroblasts and EBV-Induced Transformation

J.H. Pope *et al.*, (1974) found that the transformation of non-adherent subpopulations of cells from HPBL populations was inhibited when in culture with EBV QIMR-WIL and autochthonous fibroblasts. D.J. Moss *et al.*, (1977), investigated the phenomenon of "inhibition of EBV-induced transformation *in vitro*" more closely. They co-cultured non-adherent cells from HUCLs or HPBLs (from seropositive and seronegative donors) with EBV QIMR-WIL and autochthonous fibroblasts of either foetal or adult origin. They found that transformation was inhibited only if the autochthonous fibroblasts were co-cultured with the non-adherent cells which had been obtained from seropositive donors. They suggested that the autochthonous fibroblasts were not inhibitory to EBV-induced transformation *in vitro*. The hypothesis which was put forward was that the fibroblasts supported the expression of inhibitory factor(s) which were present among the lymphocyte populations from seropositive donors.

D.J. Moss et al., (1978) then attempted to test this hypothesis in their efforts to establish LCLs from HUCLs or HPBLs from seropositive and seronegative donors. They found that, LCLs could not be established from the lymphocyte populations which had been obtained from seropositive donors. They separated and reconstituted lymphocyte subpopulations from seropositive donors. They found that LCLs could not be established only if T-cells were present in the cultures of HPBLs which had been obtained from seropositive donors.

5.4. Thymus-derived Cells and EBV-Induced Transformation

E. Svedmyr et al., (1975) tested PBLs from IM patients and normal subjects, for their cytotoxic activity against EBV-genome positive lymphoblastoid cell lines (EBV-GP LCLs), or EBV-genome negative lymphoblastoid cell lines (EBV-GN LCLs). They found that unfractionated lymphocyte populations which had been obtained from IM patients and normal subjects were cytotoxic to EBV-GP LCLs as well as EBV-GN LCLs. On removal of the cytotoxic lymphocytes which bore complement receptors (natural killer cells), from the lymphocyte populations of IM patients, the cytotoxicity was specific for EBV-GP LCLs. On the other hand, on removal of natural killer cells from the lymphocyte populations which had been obtained from normal subjects, the cytotoxic activity was completely obliterated. They suggested that lymphocyte populations from IM patients contained EBV-specific cytotoxic cells. These EBV-specific cytotoxic cells were not present in the lymphocyte

populations of normal subjects.

T. Shope et al., (1979) separated T-cell populations from neonates, seropositive and seronegative donors into :

(1) subpopulations of T-cells which bore receptors for the Fc portion of IgG, (T_G) lymphocytes,

and

(11) subpopulations of T-cells which bore receptors for the Fc portion of IgM (T_M) lymphocytes.

They found that T_G lymphocytes which had been obtained from seropositive as well as seronegative donors, inhibited the outgrowth of autologous B-cells which had been exposed to EBV in vitro. On the other hand, T_G lymphocytes and other T-cell subpopulations which had been obtained from neonates were not inhibitory to EBV-induced transformation in vitro.

6. EXPRESSION AND CONTROL OF THE TRANSFORMED STATE

G. Miller et al., (1973) described EBV-induced transformation in vitro as :-

- cell aggregation
- acid production
- increase in cell number
- acquisition by the culture, the capacity to be repeatedly subdivided.

- Cell aggregation was the state whereby cells which had been transformed, replicated as clumps -

here it is noteworthy that BL-derived LCLs grow as single or small, loose clumps when in culture (K. Nilsson, 1978).

- Acid production was the state whereby there was a change in the pH of the growth medium from neutrality to acidity.
- Increase in cell number was the state whereby there was a significant increase in cell number as the transformed state was established.
- Acquisition by the culture to be repeatedly subdivided - as the transformed state was established the cells could be repeatedly subdivided and subcultured without loss of the characteristics of the transformed state.

6.1. Control of the Transformed State at the Transcriptional Level.

In cells which have been transformed by oncogenic viruses, a single gene or a small genetic region of the virus is sufficient to initiate and maintain the transformed state. Many viral mRNAs contain polyadenylic acid at their 3' termini which is thought to be associated with transport and stability of the RNA species. T. Orellana *et al.*, (1977) analyzed the EBV-specific RNA in cultures of productively infected cells, B958, and HR-1, and restringently infected cells Namalwa, and Raji, by hybridization of cellular RNA with purified EBV DNA. They found that B958 and HR-1 contained viral RNA which was encoded

by approximately 45% of EBV DNA. Raji and Namalwa contained RNA which was encoded by approximately 30 and 16% of EBV DNA respectively. Almost all of the viral RNA species in B958 and HR-1 was found in the polyribosomal and polyadenylated RNA fractions. In Raji and Namalwa, the polyadenylated and polyribosomal viral RNA fractions were enriched for a class of EBV RNA which was encoded by approximately 5% of EBV DNA. The same EBV DNA sequences encoded the polyadenylated and polyribosomal fractions in both Raji and Namalwa. After superinfection of Raji cells with EBV HR-1, they found that abortively infected Raji contained RNA which was encoded by at least 41% of EBV DNA. The polyadenylated RNA of superinfected Raji was enriched for a class of EBV RNA which was encoded by approximately 20% of EBV (HR-1) DNA. The polyadenylated RNA in superinfected Raji was encoded by the same DNA sequences which encoded the RNA species which was present in Raji before superinfection. They suggested that the polyadenylated RNA and polyribosomal RNA fractions of restringently infected cells could be related to the maintenance of the transformed state.

N. Raab-Traub et al., (1978) analysed the restriction endonuclease enzyme fragments of two transforming strains of EBV, EBV B958, and EBV W91, and a non-transforming EBV strain, EBV HR-1. They hybridized the DNA fragments of EBV B958, EBV W91, and EBV HR-1 by hybridization blot experiments of the three EBV DNA species. They found that EBV (HR-1) DNA was missing DNA sequences which were contained in the ECoR1

1-A, J through K and Hsu 1-B fragments of EBV (B958) DNA. The fragments which were missing in EBV (HR-1) DNA, were common to both EBV (B958) DNA and EBV (W91) DNA. They suggested that EBV (HR-1) DNA lacked the capacity to induce and maintain the transformed state due to the absence of the necessary "transforming" sequences.

6.2. Maintenance of the Transformed State at the Translational Level.

The entry of the transformed cell into the transformed state is associated with the detection of an Epstein-Barr Virus-associated Nuclear Antigen (EBNA). T. Aya et al., (1974) found that among EBV-infected cells, EBNA was detectable within two days of infection. B. Reedman et al., (1973) found that EBNA was detectable by an anticomplementary immunofluorescence (ACIF) test among productively as well as restringently infected LCLs. J. Luka et al., (1977) found that EBNA had a very strong affinity for double, as well as single stranded DNA. They suggested that EBNA may contribute to transformation by binding to DNA and thereby influence transcription and/or DNA replication. But, D.L. Thorley-Lawson et al., (1978) found that EBNA appeared on the DNA transcription machinery only when the cell and its DNA had entered the transformed state. When HPBLs were cultured with EBV B958 in the presence of phosphonoacetic acid, EBNA and cellular DNA synthesis occurred. On the other hand, neither EBNA nor cellular DNA synthesis occurred in the presence of cytosine arabinoside.

A.T. Powell et al., (1979) hybridized EBV (B958) DNA which was homologous to Namalwa polyadenylated or non-polyadenylated RNA, to blots containing ECoR 1, Hsu 1, or ECoR 1/Hsu 1 double cut fragments of EBV (B958) DNA or EBV (W91) DNA. They found that the viral polyadenylated RNA fractions from Namalwa cells was encoded by DNA which was contained primarily in the Hsu 1-A/ECoR 1-A and Hsu 1-B/ECoR 1-A fragments. The molecular weight of EBNA has been determined to be approximately 174×10^3 daltons. The purified molecule has been found to dissociate into two components of 98×10^3 daltons each (Matsuo et al., 1979 and J. Luka et al., 1978). R. Pritchett et al., (1975) found that the molecular weight of EBV DNA in producer cells was 1×10^8 daltons. T. Lindahl et al., (1976) found that the EBV DNA in Raji had a molecular weight similar to that of the virus. A.T. Powell et al., (1979) have suggested that the transformed state could be maintained at the translational level. EBNA could be encoded by 2×10^6 daltons of EBV DNA.

6.3. Herpesvirus papio and EBV-associated Nuclear Antigen

L. Falk et al., (1976, 1977) carried out hybridization experiments on Herpesvirus papio (HVP) with EBV-complementary RNA (cRNA). They found that EBV DNA and HVP DNA had 40-50% homology. HVP-infected baboon lymphocytes had B-cell characteristics and sera from seropositive baboons had cross-reactivity with sera from human EBV seropositive subjects. However, they could not detect a nuclear antigen in HVP-

genome positive (GP) LCLs, *in situ*. S. Ohno *et al.*, (1977b) extracted nuclear fractions from HVP-GP LCLs. They added the nuclear extracts to methanol/acetic acid-fixed chicken red blood cells (CRBCs) or cells from EBV-GN LCLs, BJAB and Ramos. They found that a nuclear antigen could be detected in CRBCs, BJAB, and Ramos, by the acid-fixed nuclear-binding (AFNB) technique when coupled with ACIF. S. Ohno *et al.*, (1978) found that there was an asymmetric cross-reactivity between human EBNA positive antisera and HVP nuclear antigen (HUPNA)-positive antisera. All the EBNA positive antisera reacted with nuclear extracts from productively and restringently infected HVP-GP LCLs. On the other hand, none of the antisera which had been obtained from baboons which were reactive in the AFNB technique for HUPNA, were reactive in the ACIF for EBNA when tested against EBV-GP LCL, Raji. Approximately half of the anti-HVP sera reacted with HUPNA.

G. Klein *et al.*, (1978) superinfected BJAB and Ramos and the restringently infected HVP-GP LCL, 26CB-1, with EBV B958. They also superinfected Raji, BJAB and Ramos with HVP from the HVP, productively infected LCLs, 9B and 18-C. They found that EBNA could be detected in 26CB-1, *in situ* and EA and VCA could be detected in Raji. EA could be induced in BJAB and 26CB-1, but EBNA could not be detected in Ramos, BJAB or 26CB-1. They suggested that the lack of *in situ* detection of a nuclear antigen among HVP-infected cells, was not due to an innate inability of the virus to induce the synthesis of the nuclear antigen.

Man and baboon are 20 x 10^6 years apart. EBV and HVP could have originated from a common ancestor. If HVP is the more primitive of the two virus strains, perhaps HVP-infected baboon cells can be transformed and the transformed state maintained without the continual synthesis of a virally induced product. On the other hand, it is probably essential that EBV-infected cells continually translate a virally induced product for the maintenance of the transformed state.

7. EBV-TRANSFORMED HUMAN UMBILICAL CORD BLOOD LYMPHOCYTES

D.J. Moss et al., (1975) and T. Aya et al., (1974) found that EBV-transformed HPBLs and HUCLs produced EBNA but could not support the replication of other virally induced products, EA, MA and VCA. The inability of EBV-infected HUCLs to support the syntheses of most virally induced products is not known. However, the evidence is emerging that it could be associated with the number of EBV genome copies/cell (EBV GCs/C).

G. Klein et al., (1976) EBV-converted EBV-GN LCLs, BJAB and Ramos into EBV-GP LCLs, BJAB (P_3HR-1) and Ramos (P_3HR-1), by superinfection with EBV P_3HR-1. After induction of EA in BJAB (P_3HR-1) and Ramos (P_3HR-1) with 5-iododeoxyuridine they found that the inducibility of EA varied among the converted lines. They suggested that there was a possible relationship between the susceptibility of cells to EA induction and the number of EBV GCs/C.

K. Bister et al., (1979) treated clones of Raji cells which had various numbers of EBV GCs/C with 12-O-tetradecanoylphorbol-13-acetate (TPA). They found that the susceptibility of clones to EA induction was dependent on the number of EBV GCs/C. Clones with a higher number of EBV GCs/C were more susceptible to EA induction by TPA, than clones with a lower number of EBV GCs/C. H. zur Hausen et al., (1978) found that TPA efficiently induced the synthesis of EA and VCA in B958 and EA in Raji and NC_{37}. On the other hand, EBV-transformed HUCLs were completely unsusceptible to either EA or VCA induction. J. Hudewentz et al., (1980) induced EA in Raji and then determined the amount of viral DNA/cell, by measuring the reassociation kinetics of labelled EBV DNA from virus particles, in the presence of EBV DNA from Raji cells. They found that the amount of EBV DNA/cell remained constant after EA induction by TPA. This suggested that TPA did not increase the amount of EBV DNA/cell among restringently infected cells. I. Ernberg et al., (1977) determined the average number of EBV GCs/C by EBV-cRNA and measured the amount of EBNA/cell by quantitative immunofluorimetry. They then compared the two parameters and found that the greater the number of EBV GCs/C, the greater the fluorescence intensity.

7.1. Epstein-Barr Virus Copies Per Cell

H. zur Hausen et al., (1972) found that the numbers of EBV GCs/C was a constant feature of EBV-GP LCLs. Although

the numbers varied from one cell line to another, it remained constant for each cell line, between passages. They found that the numbers varied between 3 and 200 for LCLs which had been established from NPC, BL, IM, and HPBL. On the other hand, LCLs which had been established from HUCLs contained no complete EBV GC/C as determined by hybridization of EBV-cRNA. T. Lindahl et al., (1974) found that African BL-derived LCLs contained between 10 and 100 EBV GCs/C. J.S. Pagano et al., (1976) found that LCLs which had been established from IM patients contained 20 to53 EBV GCs/C while LCLs which had been established from HUCLs, contained only 10 to 15 EBV GCs/C.

7.2. IMMUNOGLOBULIN CLASSES, SUBCLASSES AND AGE

7.2.1. Distribution of Subclasses Among Immunoglobulin Proteins.

L. Hood et al., (1967) obtained pools of sera which had been prepared from 20 different vertebrate species, and pools of human and murine myeloma proteins. Using gel-electrophoresis, they analyzed the distribution of kappa (k) and lambda (λ) Ig subclasses among the different Ig protein fractions which had been obtained from the sera and myeloma samples. They found that the common (c) region of the Ig subclasses which had been obtained from closely related animals, were generally more similar in their amino acid sequences than those which had been obtained from distantly related animals. Among adult mice and humans, they found that the k : λ ratios were 95 : 5% and 60 : 40% respectively.

They suggested an evolutionary relatedness between the genes which controlled the syntheses of Ig(k) subclasses among human and mice species. They found that 60% of the amino acid residues which had been obtained from human and mice, Ig(k), c-region proteins, were similar in their sequence distribution.

7.2.2. Immunoglobulin Subclasses Among Lymphocyte Populations

G. Haughton et al., (1978) examined the distribution of sIg-positive cells among the lymphocyte populations of mice of varying ages by indirect IF. They prepared lymphocyte populations from the spleens, bone marrows, and livers. They found that foetal liver, bone marrow, and spleens contained sIg-positive cells with a k : λ ratio which approximated equality. Among lymphocyte populations which had been obtained from adult spleens, there was a dominance of sIg(k)-bearing cells over sIg(λ)-bearing cells. The dominance increased as a function of age. They hypothesised that the preponderance of sIg(k)-positive cells in adult mice had been established by an antigen driven clonal expansion of the repertoire of Ig(k) genes which expanded as a function of age.

K.K. James et al., (1974) found that the ratio of k : λ Ig subclasses among the lymphocyte populations which had been obtained from children (< 10 yrs), approximated equality. On the other hand, among the lymphocyte populations which had been obtained from human adult subjects, the number of

sIg(k)-bearing cells exceeded the number of sIg(λ)-bearing cells in an approximate ratio of 2:1.

7.2.3. Immunoglobulin Classes Among Human Lymphocyte Populations.

P. Lobo et al., (1975) determined the %ages of sIg-positive cells among lymphocyte populations which had been obtained from human subjects. Among populations which had been obtained from the peripheral blood of 17 healthy adult subjects, they found that 18% were sIg^+. However, this % age was reduced to 9 when the lymphocytes were pretreated by washing, followed by incubation at 37°C, prior to the IF test. This suggested that the lymphocyte populations from HPB contained an equal number of $sIg(\mu)^+$ and $sIg(\gamma)^+$ cells.

S.E. Froland et al., (1971) used antisera which had been prepared against the $F(ab)_2$ fractions of the antibody molecules, to investigate the distribution of sIg heavy chain classes among HPBL populations. They found that the %ages of $sIg(\mu)^+$, $sIg(\gamma)^+$, and $sIg(\alpha)^+$, cells were; 6 (range 3-12), 5 (range 2-7), and < 1 respectively. K.K. James et al., (1974) looked at the distribution of sIg heavy chain classes among lymphocyte populations which had been obtained from HPB and HUCB. Among HPBL populations of children (< 10 yrs), $sIg(\mu)^+$ cells exceeded the number of $sIg(\gamma)^+$ cells. Among HUCL populations which had been obtained from 12 subjects, the %ages of $sIg(\mu)^+$ and $sIg(\gamma)^+$ cells were 13 and 5-9 respectively. Among lymphocyte populations which had been obtained from adults, the %ages of $sIg(\mu)^+$ and $sIg(\gamma)^+$ cells approximated equality.

7.2.4. Lymphoblastoid Cell Lines : Immunoglobulin Classes and Subclasses.

C.M. Steel et al., (1977) investigated the phenomenon of Ig classes and subclasses by haemagglutination inhibition assay, in relation to EBV-transformed LCLs which had been established from HUCLs and HPBLs. They found that among EBV-transformed HUCLs, only Ig(μ) heavy chain class was secreted. On the other hand, among EBV-transformed HPBLs (adolescents and adults), Ig(μ), Ig(γ) and Ig(α) heavy chain classes were secreted. While the ratios of k : λ among LCLs (HUCLs), was 1:1, the ratios of k : λ among LCLs which had been established from HPBLs were 1.4 : 1.0, and the ratios of Ig(μ), Ig(γ), Ig(α) among LCLs (HPB) were 2:1 : 0.5 respectively.

8. EPSTEIN-BARR VIRUS : SEROLOGY

8.1. The Acquisition of EBV Seropositivity

Most primary infections of EBV occur in childhood and early adolescence and are accompanied by permanent sero-conversion (seropositivity) and life-long harbouring of the virus. The acquisition of EBV seropositivity during primary infections, confer permanent immunization against IM. EBV is transmitted vertically and is expressive as IM mainly when contracted during late adolescence. The acquisition of EBV seropositivity does not necessarily lead to the expression of any EBV-associated disease. W. Henle et al., (1970c) found that among patients who had

undergone surgery with extracorporeal circulation with fresh blood and transfusions of stored blood, six without preoperative antibodies, developed antibodies to EBV without expressing any overt symptoms for NPC, IM or BL.

The titres and types of antibodies which are directed against the EBV-associated antigens, vary among seropositive subjects.

W. Henle et al., (1970a) found that among NPC patients, there was a high titre of antibodies to MA, EA and VCA. P. Gunven et al., (1970) found that BL patients had elevated titres to MA and VCA. I. Ernberg et al., (1975) suggested that high titres to EA in long-term BL survivors, was indicative of tumour recurrence. W. Henle et al., (1970b) suggested that antibodies to EA possibly reflected current or recent disease processes which were associated with EBV. W. Henle et al., (1971) found that, seropositive subjects who had recovered from IM had antibodies to MA, VCA, and EBNA, but not EA.

8.2. Antibodies to Early Antigen

G. Henle et al., (1971) found that EA could be detected as two distinct forms in IF experiments. The restricted (R) form occurred as aggregates of varying sizes in the cytoplasms of infected cells. The diffused (D) form diffused throughout the nuclei and cytoplasms of infected cells and sometimes spread as a fluorescent component to adjacent cells in the smears. G. Henle et al., (1971); W. Henle et al., (1971); and W. Henle et al., (1973) found that : about 70% of IM

patients had antibodies to the (D) component of EA; and 80% of BL and NPC patients had anti-EA antibodies. While anti-R was dominant in BL, anti-D was dominant in NPC patients.

8.3. Membrane Antigen and the Viral Envelope

D. Silvestre et al., (1974) found that MA could be detected as two forms. Early membrane antigen (EMA) was found to be an early, and late membrane antigen (LMA) was found to be a late product of the EBV DNA synthetic machinery. Both EMA and LMA were found to be detectable on the viral envelopes as well as on the membranes of productively infected cells. I. Ernberg et al., (1975) found that EMA and EBNA were the only EBV-associated antigens which were detectable among the tumour cells which had been obtained from NPC and BL patients. L. Gergely et al., (1971) suggested that the MA complex on the surfaces of infected cells could represent viral envelope components which are inserted into the cell membranes. They found that exposure of EBV to sera with high anti-MA titres, neutralized the effects of the virus when judged by the inability of EBV (P_3HR-1) to induce EA in Raji cells. The virus neutralizing titres could be reduced if the sera were preabsorbed with producer cells which had a high, but not with producer cells which had a low frequency of MA positive cells.

J.R. North et al., (1980) radioiodinated the MA complex on the surfaces of B958, M-ABA, QIMR-WIL, and P_3HR-1 producer

cells. They analyzed the polypeptides which had been obtained, by immunoprecipitation of the MA-associated proteins and SDS-polyacrylamide gel-electrophoresis. They compared their gel profiles from MA on the cell surfaces with gel profiles from the viral envelopes. They found that MA appeared as a complex of four polypeptides both on the cell surfaces as well as on the viral envelopes. Additionally, they found that the two largest polypeptides showed minor differences in mobilities. The differences were dependent on the origin of the virus strains i.e. whether they had been passaged in marmoset or human cells.

8.4. Rheumatoid Arthritis Nuclear Antigen (RANA)

M.A. Alspaugh et al., (1978) found that sera which had been obtained from some rheumatoid arthritic (RA) patients, contained antibodies which had been directed against a nuclear antigen (Rheumatoid arthritis nuclear antigen) in producer cells, Wil_2. They found that normal HPBLs did not contain Rheumatoid arthritis nuclear antigen (RANA), but after infection with EBV showed an increased frequency as the cells established the transformed state.

8.4.1. RANA and Epstein-Barr Virus Nuclear Antigen

T. Aya et al., (1974) found that the frequency of EBNA positive cells increased as the cells established the transformed state. S. Slovin et al., (1980) found that RANA could be detected in productively as well as

restringently infected cells Wil_2 and Raji. B. Reedman et al., (1973) showed that EBNA could be detected in restringently as well as productively infected cells. B. Reedman et al., (1975) suggested that the low level of EBNA which is present among EBV-GP LCLs, precludes its detection by IF. M.A. Catalano et al., (1979) observed that among RA patients, there was a higher than normal titre against EBNA. M.A. Alspaugh et al., (1978) have shown that RANA can be detected by IF. M.A. Alspaugh et al., (1978) have suggested that the main component of the RA complex is IgG. A. Liabeuf et al., (1975) found that the main component of anti-EBNA antisera which facilitates the detection of EBNA in ACIF, is IgG. S. Slovin et al., (1980) found that in synchronized Raji and Wil_2 cells, EBNA, and RANA could be detected at specific but close stages of the cell cycle. RANA reached a maximal level during early G_1, declined during late G_1, when EBNA was at its maximal level. However, RANA reappeared during late G_2/M, when EBNA could not be detected.

One cannot therefore help the speculation on an association between the Rheumatoid arthritis nuclear antigen, EBNA, and their associations with Rheumatoid arthritis.

8.5. The Soluble (S) Antigen

V. Vonka et al., (1970a) examined human sera for antibodies to complement-fixing (CF) antigens which had been obtained from EBV-transformed LCLs. They investigated a

soluble (S) antigen and a partially purified EBV particle (V) antigen from cell extracts. They compared two parameters; the CF assay, and the indirect EBV-specific IF. They found that all antisera which were reactive with the (S) antigen were also reactive with the (V) antigen. There was a high correlation between the CF-V antibody titre and the EBV-specific IF titre with producer cell line EB-3. Many CF-S antibody negative antisera had high CF-V antibody titre. They suggested that the antigens which were detectable by EBV specific IF and the CF-V antigens, were closely related. On the other hand, the CF-S antigen was immunologically distinct.

8.5.1. (S) and Epstein-Barr Virus Nuclear Antigen

V. Vonka et al., (1970b) found that cellular extracts which had been derived from restringently as well as productively infected cells contained the (S) antigen. B. Reedman et al., (1973) suggested that (S) and EBNA could be related.

G. Lenior et al., (1976) characterized (S) which had been obtained as cellular extracts from Raji, by sucrose gradient centrifugation, gel filtration, and ion exchange chromatography. They found that (S) had a molecular weight of 180×10^3 daltons and fractions which contained (S), inhibited the ACIF test for EBNA. J. Luka et al., (1978) purified EBNA from cellular extracts of Raji, B958, and Namalwa, by DNA-cellulose chromatography, hydroxyapatite chromatography and gel filtration. They found that, all

EBNA preparations inhibited the ACIF test for EBNA when coupled with the complement fixation assay for (S). The cellular extracts bound to methanol/acetic acid-fixed (AFNB) metaphase chromosomes. The purified extract had a molecular weight of 170 x 10^3 to 200 x 10^3 daltons.

S. Ohno et al., (1977a) extracted, purified (S) by DNA-cellulose chromatography, and combined AFNB with ACIF. They found that a brilliant nuclear staining, characteristic of EBNA, could be detected. The purified antigen was found to have a molecular weight of 174 ($\pm$ 15) x 10^3 daltons, as determined by sucrose gradient centrifugation and gel filtration experiments. They suggested that (S) and EBNA were identical.

9. THE EPSTEIN-BARR VIRUS DNA

9.1. EBV DNA In Producer Cells.

D. Given et al., (1978) determined the arrangements of restriction endonuclease cleavage sites of EBV (B958) DNA, EBV (W91) DNA and EBV (HR-1) DNA. They used ECoR 1, Hsu 1 and Sal 1 to determine the sizes and homologous sequences of the EBV DNAs. They used EBV (B958) DNA (Fig. 1) as the prototype to relate their findings to EBV (W91) DNA and EBV (HR-1) DNA.

They found that the sizes of almost all the ECoR 1, Hsu 1 and Sal 1 fragments of EBV (W91) DNA and EBV (B958) DNA were identical. A single ECoR 1 fragment, ECoR 1-C, was approximately 7 Megadaltons (MDs) larger than the ECoR 1-C fragment of EBV (B958) DNA. On digestion of the region

of EBV (W91) DNA which corresponded to ECoR 1-C with Hsu 1 or Sal 1, they obtained two fragments; Hsu 1 (D_1/D_2) and Sal 1 (G_2/G_3). The total sizes of Hsu 1 (D_1/D_2) or Sal 1 (G_2/G_3) was 7 MDs larger than the ECoR 1-C fragment from EBV (B958) DNA. They labelled EBV (HR-1) DNA and removed the sequences which were homologous to EBV (B958) DNA. They found that the ECoR 1-C fragment of EBV (HR-1) DNA was similar in size to the ECoR 1-C or Hsu 1 (D_1/D_2) fragments of EBV (W91) DNA. The ECoR 1-C fragment of EBV (Jijoye) DNA was approximately 7 MDs larger than the ECoR 1-C fragment of EBV (B958) DNA and was identical in size to the ECoR 1-C fragment of EBV (W91) DNA. They implicated a similarity in structure and sequence between EBV (HR-1), EBV (W91), and EBV (Jijoye) DNAs. They suggested that the similarity between the "extra" EBV DNA which had been found in EBV (HR-1) DNA, EBV (W91) DNA and EBV (jijoye) DNA could reflect a characteristic sequence and structure of BL-derived EBV strains.

9.2. Sequence Variability Among EBV DNAs

E. Henderson et al., (1977) found that the frequency of transformation of HPBLs was 100 times less sensitive, and lymphocytes which had been obtained from the peripheral blood of marmosets was 1,000 times less sensitive, than HUCLs to EBV B958-induced transformation. L. Rymo et al., (1979) used the restriction endonuclease enzyme ECoR 1 to fragment intracellular EBV DNAs from 23 different isolates of LCLs which had been established from ; HUCLs, HPBLs,

BL, NPC and IM subjects. They found that sequence variability among the different DNA isolates was largely confined to the ECoR 1-C, -A, and -I fragments when they related their fragment analysis to EBV (B958) DNA linkage map, Fig. 1. They found that EBV (883-L) DNA was completely devoid of the ECoR 1-C fragment. They suggested that, the possibility could not be eliminated that, a small, variable region of host DNA may be included in the ECoR 1-C fragment of intracellular EBV DNA.

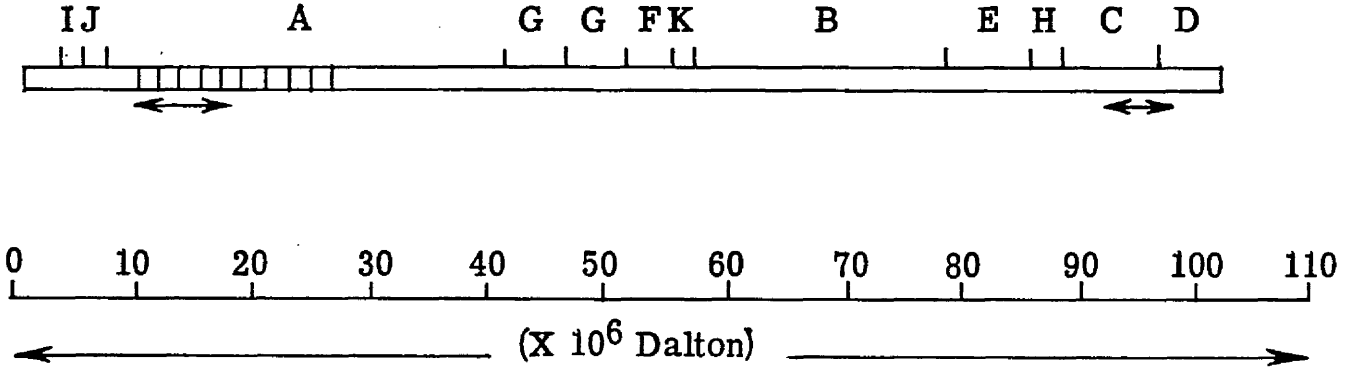
9.2.1. Amplification of EBV DNA

B. Sugden et al., (1979) infected HPBLs with 0.02 to 0.10 EBV (B958) DNA particles/cell and immediately cloned the cells in agarose. They found that within 30 generations, all the transformed clones contained between 5-800 EBV GCs/C. They related the amount of intracellular EBV DNA, X, to the number of EBV GCs/C, Y. They found that the greater the value of X, the higher the value of Y. They suggested that EBV DNA could have undergone amplification (relative to cellular DNA) after transformation had occurred.

If amplification of EBV DNA does occur (relative to cellular DNA) in transformed cells, it is conceivable that the heterogeneity of the ECoR 1-A, -C, and -I, could be realized.

Fig. 1. Linkage map of ECoR1 fragments of EBV (B958) DNA

The sites of endonuclease cleavage are indicated by perpendicular lines above the open bar, denoting the linear EBV (B958) DNA molecule. The lines within the open bar indicate the repeated sequences which are present in the A fragment and the terminal redundant regions. The lower scale denotes the size in terms of the molecular weight of the double stranded DNA molecule.



(Reproduced from L. Rymo et al., 1979)

9.3. Circular EBV DNA

M. Nonoyama et al., (1972) found that although the EBV DNA in Raji cells was detected within the cellular chromosomes, EBV DNA could be separated from cellular DNA on alkaline sucrose gradients, as complete, or nearly complete strands. They suggested that EBV DNA in restringently infected cells was not bound to chromosomal DNA as integrated sequences, but existed as unintegrated replicons. A. Adams et al., (1973) partly agreed with the findings of M. Nonoyama et al., but they found that a fraction of EBV DNA in Raji could not be separated from cellular DNA in neutral glycerol gradients. A. Tanaka et al., (1974) found that 80-90% of EBV DNA in Raji was not integrated but 10-20% remained bound to cellular DNA. The unintegrated, purified, EBV DNA had a density of 1.716 - 1.717 gm/cm^3 as determined by caesium chloride equilibrium centrifugation. A. Adams et al., (1975) coupled sedimentation analysis on neutral glycerol gradients by hybridization experiments using EBV-cRNA. They recovered two distinct species of DNA which sedimented at rates of 1.70 - 1.75 and 1.10 - 1.12 times as fast as linear EBV DNA from producers. They suggested that the intracellular EBV DNA in Raji had the hydrodynamic properties of a covalently closed, circular, and a nicked, (containing single strand breaks) circular form. The intracellular EBV DNA in Raji could therefore replicate as integrated, as well as an unintegrated molecule.

B. Hampar et al., (1974) found that the circular EBV DNA in Raji cells replicated only during the early synthetic (S_1) phase of the cell cycle, suggesting that the replication of the EBV (circular) DNA is indeed entwined with the synthesis of cellular DNA. C. Kintner et al., (1979) digested EBV (B958) DNA with λ-exonuclease and found that the DNA molecule, upon reannealing formed full length circles. They suggested that the viral DNA could contain repeat sequences at its termini which permitted the circularization of the molecule.

MATERIALS

1. Fine Chemicals

The following chemicals were supplied as indicated :-

Fluorescein isothiocyanate (FITC), Isomer I.	Sigma (U.K.) Ltd., London, England.
Tris (hydroxymethyl) aminoethane (Trizma base)	"
Triton X-100, purified	Koch-Light Labs. Ltd., Colnbrook, England.
N',N'-methylene bisacrylamide	Eastman Kodak Co., Rochester, New York.
Heparin, preservative free	Evan's Medical Ltd., Liverpool, England.
Giemsa staining solution	Gurr Products, Suffolk, England.
Evan's blue, dry powder	"
Trypan blue, dry powder	"
DPX mounting medium	12, The Viaduct, Middlesex, England.
Ficoll 400	Pharmacia Fine Chemicals, Sweden.
Sodium hypaque	Winthrop Labs., Surrey, England.

2. Cell Culture Materials

The following cell culture materials were supplied by :-

Gibco-bio-Cult (Europe) Ltd., Washington Road, Paisley, Scotland.

RPMI-1640, dry powder

Foetal calf serum

Screw-capped (10 ml) plastic tubes

Falcon tissue culture flasks (25 cm^2, 75 cm^2) and petri dishes (50 mm in dia.).

Freeze-dried stocks of streptomycin sulphate and benzyl penicillin were supplied by :-

Glaxo Pharmaceuticals, London, England.

Glass test tubes, and pipettes were supplied by :-

Corning Glass Works, Corning, New York, U.S.A.

3. Radiochemicals.

The following radiochemicals were supplied by :-

The Radiochemical Centre, Amersham, England.

Amino Acids	Specific Activity
L-[4, 5-^{3}H] leucine	61 Ci/mMole
L-[^{35}S] methionine	1,280 Ci/mMole

4. Photographic and Liquid Scintillation Spectrometry Materials

The following materials were supplied as indicated :-

Ektachrome 400 film	Kodak (U.K.) Ltd., London, England.
Ektachrome 50 film (Tungsten Grade)	"
X-Omat H film, 24 x 30 cm	"
DX-80 Developer	"
FX-40 X-Ray Liquid Fixer	"
Lightproof vinyl film bags	H.A. West (X-Ray) Ltd., Edinburgh, Scotland.
2,5 Diphenyloxazole (PPO)	Koch-Light Labs. Ltd., Colnbrook, England.
Transparencies from Ektachrome 400 and 50	Brian and Spears Colour Labs., Glasgow, Scotland.

Other materials not mentioned above, were supplied by BDH Chemicals Ltd., Poole, Dorset, England.

5. Lymphoblastoid Cell Lines

The following lymphoblastoid cell lines were obtained as a kind gift from :-

Dr. G.B. Clements, Institute of Virology,
University of Glasgow.

Bec-11, Daudi, B958, QIMR-WIL, Raji (6HAT25DC)

6. Laboratory Bred Animals

Mice (Balb/c) were bred in the animal colony of The Biochemistry Department, University of Glasgow, by brother/sister (F_1) matings.

7. Antisera and Antibody Preparations

The following antisera which were raised against human immunoglobulins were supplied as indicated :-

FITC-conjugated Rabbit anti-IgM (μ-specific)	Behring Hoechst Pharmaceuticals, Hoechst House, Hounslow, England.
FITC-conjugated Rabbit anti-IgG (γ-specific)	"
FITC-conjugated Rabbit anti-λ (Bence Jones Protein)	"
FITC-conjugated Rabbit anti-k (Bence Jones Protein)	"
Rabbit anti-IgG (γ-specific)	Miles Labs., Research Products Ltd., Slough, England.
Rabbit anti-k (Bence Jones Protein)	"
Rabbit anti-λ (Bence Jones Protein)	"
Rabbit IgG (Rb IgG)	Nordic Immunological Labs. Ltd., Berks., England.

Human antisera which contained antibodies directed against EBV-associated antigens were obtained as a gift from :-

Dr. George Klein, Karolinska Institutet,

Stockholm, Sweden.

The relevant information related to the special features of anti-EBV antisera is displayed in Table 1.

In addition, various antibody preparations (Section 10, Methods) were derived by :-

Mrs E. Blakeley, Biochemistry Department,

University of Glasgow.

8. Chromatography and Other Materials

Sepharose 4B	Pharmacia (GB) Ltd., London, England.
Sephadex; G-200, G-100, A-50	"
Cytocentrifuge papers	Shandon Ltd., Camberley, England.
Microscope glass slides and coverslips	Chance (Propper) Ltd., Warley, England.
Millipore filters	Millipore (U.K) Ltd., London, England.
Amicon filters	Amicon Corporation, Lexington, Mass., U.S.A.

Table 1. Characteristics of Human anti-EBV Antisera

Designation	Antigen Titre				*IF
	EA		VCA	EBNA	Reactivity
	D	R			
Ingela	<10	<10	<10	<10	$EBNA^-$
Ingemar	<10	<10	<10	<10	$EBNA^-$
Eva Klein	<10	<10	<10	<10	C'
Walter Owino	40	320	320	160	$EBNA^+$
$F_{3/4}$-Esther	2560	ND	5120	ND	$(EA/VCA)^+$
F_1-Katana	-	-	640	ND	VCA^+

Abbreviations : F_1, F_3, F_4 = FITC conjugate number

*-, +, C' = Negative, Positive, Complement

ND = Not done.

METHODS

1. STOCK SOLUTIONS

1.1. Cell Culture Medium

The components of RPMI-1640 (the cell culture medium which was used throughout this work), are displayed in Table 1. The solution was prepared from the dry powder in deionised water and the pH monitored to 7.0 with concentrated HCl. The medium was sterilized by Millipore filtration (pore size, 0.22 μm) and stored at 4^{o}C. The complete medium (CM) was prepared according to the following formulation :-

Complete Medium

RPMI-1640	90% (by volume)
Foetal calf serum (FCS)	10% (by volume)
Benzyl penicillin	100 i.u./ml
Streptomycin sulphate	100 μg/ml
L-glutamine	0.3 mg/ml

The components of complement in FCS were inactivated by heating the FCS at 56^{o}C for 30 mins.

Stock solutions (X10) of benzyl penicillin/streptomycin sulphate, and L-glutamine were stored at -20^{o}C.

Table 1 Composition of RPMI-1640 Cell Culture Medium

Amino Acids	mg/l
L-arginine	200.00
L-asparagin H_2O	56.82
L-aspartic acid	20.00
L-cysteine, disodium salt	59.15
L-glutamic acid	20.00
L-glutamine	300.00
glycine	10.00
L-histidine	15.00
L-hydroxyproline	20.00
L-isoleucine	50.00
L-lysine HCl	40.00
L-leucine	50.00
L-methionine	15.00
L-phenylalanine	15.00
L-proline	20.00
L-serine	30.00
L-threonine	20.00
L-tryptophan	5.00
L-tyrosine	20.00
L-valine	20.00

Table 1 (contd.)

Vitamins	mg/l
Biotin	0.20
D-calcium pantothenate	0.25
Choline chloride	3.00
Folic acid	1.00
i-Inositol	35.00
Nicotinamide	1.00
p-Aminobenzoic acid	1.00
Pyridoxine HCl	1.00
Riboflavin	0.20
Thiamin HCl	1.00
Vitamin B_{12}	0.005
Inorganic Salts	
Calcium nitrate	69.49
Potassium chloride	400.00
Magnesium sulphate	100.00
Sodium chloride	6,000.00
*Sodium bicarbonate ($NaHCO_3$)	2,000.00
di-Sodium hydrogen phosphate	800.00
Other Components	
Glucose	2,000.00
Glutathione	1.00
Sodium phenol red	5.00

* $NaHCO_3$ (dry powder) was added to the dissolved RPMI-1640.

1.2. Phosphate Buffered Saline, pH 7.2

Phosphate buffered saline (PBS-A) consisted of :-

	g/l
Sodium chloride	10.00
Potassium chloride	0.25
di-Sodium hydrogen orthophosphate	1.44
Potassium di-hydrogen orthophosphate	0.25

The solution was stored at room temperature.

2. GENERAL METHODS

2.1. Preparation of Smears : Cytocentrifugation

Cytocentrifuge papers were aligned with microscope glass slides (76 x 26 mm) and cytocentrifuge buckets, in the Shandon cytocentrifuge. Cells were washed in, and resuspended in, PBS-A. Aliquots (500 μl) were placed in the buckets and smears (4-5 x 10^4 cells) prepared by centrifugation at 170 xg for 10 mins. The smears were air-dried and fixed.

2.2. Trypan Blue Exclusion Test

Any clumps of cells which were in the samples of cell suspensions were completely disrupted. Cell suspensions were added to equal volumes (200 μl) of Trypan blue solution, final concentration 0.005% w/v in PBS-A.

Trypan Blue Staining Solution

Trypan blue (dry powder) was
dissolved (0.01% w/v) in PBS-A.

The suspensions of cells were left at room temperature for 10 mins. Viable cells excluded, nonviable cells incorporated the Trypan blue solution. The total number of cells was counted in a Neubauer type hemocytometer and the %age viability of the cell suspensions determined as follows :-

$$\%\text{age viability} = \frac{\text{No. viable cells}}{\text{Total No. of cells}} \times 100$$

2.3. Giemsa's Staining Technique

All steps were carried out at room temperature. Cell smears were prepared by cytocentrifugation and fixed in methanol for 10 mins. The smears were washed in distilled water and then emersed in Giemsa's staining solution.

Giemsa's Staining Solution

50% (v/v) of stock solution
in distilled water.

The smears were left for 20 mins, decolourised in acetic acid (1% v/v)/in distilled water for 5-10 mins, dehydrated in methylated spirit for approximately 5 mins and in absolute alcohol for a further 5 mins. The smears were emersed in two separate changes of xylene (5-10 mins) and mounted in DPX mounting medium with glass coverslips

(13 mm in dia.). Smears were observed by ordinary light microscopy, using Leitz Ordinary Light Microscope and photographed when necessary, with Ektachrome 50 (Tungsten grade).

2.4. Acquisition of Newborn Mice (J. Ebling et al., 1971)

Platinum loops were sterilized by flame and cooled in clean water. The loops were inserted into the vaginal passages of mice and contents of the vagina placed on microscope glass slides. Using ordinary light microscopy, the vaginal smears were examined for the presence of nucleated epithelial cells which signified the pro-oestrus stage of the oestrus cycle. Mice which were in the pro-oestrus stage were mated, and litters (4-6 mice) obtained 19-25 days after mating. Single cell suspensions were prepared from the livers and spleens of mice which were <24 hrs old.

3. Preparation of EB Virus Suspensions (T. Katsuki et al., 1975 and U. Schneider et al., 1975)

The viability of cultures from 3 day-old cultures of B958 or QIMR-WIL producer cell lines was determined by the Trypan blue exclusion test. Cells from cultures which were >90% viable were suspended in CM ($3\text{-}4 \times 10^5$ cells/ml). The cells were incubated at $37^{\circ}C$ for 9 days in 75 cm^2 tissue culture flasks. Supernatants from a total volume of 3 litres of culture was collected by centrifugation at 450 xg for 40 mins. The cell-free

extracts from B958 (EBV B958) or QIMR-WIL (EBV QIMR-WIL) were twice frozen and thawed, and centrifuged at 450 xg for 20 mins. EBV B958 and EBV QIMR-WIL were filtered by Millipore filtration (pore size, 0.8 μm in dia.) to purify the extracts and stocks stored at -70°C in aliquots of 2.0 mls.

4. ELECTRON MICROSCOPY

4.1. Fixation of Cells

Cell suspensions from 9 day-old cultures of B958 or QIMR-WIL producer cell lines were centrifuged at 350 xg for 30 mins. Gluteraldehyde solution in Sörensen's phosphate buffer (SPB) was added and the cells incubated at 4°C for 24 hrs.

Sörensen's Phosphate Buffer, pH 7.38

Sodium phosphate dibasic (0.947% w/v)	20% (v/v)
Potassium acid phosphate (0.907% w/v)	80% (v/v)

2% Gluteraldehyde Solution in SPB

Gluteraldehyde stock solution	8 ml
SPB	92 ml

The cells were then washed in three separate changes of SPB for 20 mins each wash, at 4°C. Cells were "post-osmicated" by the addition of osmium tetroxide for 45 mins.

Osmium Tetroxide in SPB

Equal parts of Osmium tetroxide (2% in distilled water) and SPB.

The cells were washed in three separate changes of SPB for 20 mins each wash, then dehydrated.

4.2. Dehydration of Cells

The cells were processed in the following solutions (1.0 ml) as indicated :-

Dehydrating Solution		Time (Mins)
Ethyl alcohol	25% (v/v)	10
" "	50% (v/v)	10
" "	75% (v/v)	10
Absolute alcohol -----	1	30
" " -----	2	30
" " -----	3	30
" " -----	4	30
Propylene oxide -----	1	10
" " -----	2	10

Araldite mixture (1.0 ml) was added to the cells, and then left at room temperature for 2 hrs. Equal parts of Araldite mixture was then added, to give a total concentration of 3 parts Araldite mixture to 1 part Propylene oxide ---2. The cells were left at room temperature for 2 hrs, and washed in Araldite mixture.

Araldite Mixture

Araldite resin (CY 212)	46.5% (by volume)
Dodecenylsuccrinic anhydride	46.5% (by volume)
Dibutyl phtholate	4.65% (by volume)
2,4,6-tri(dimethylaminoethyl) phenol	2.325% (by volume)

The dehydrated cell pellets were embedded in Araldite/ in gelatin at 60°C for 20 hrs, the gelatin material subsequently dissolved in hot water and the cell pellets sectioned by Electron Microscopy.

4.3. Sectioning of Cell Pellets

EM grids (mesh size 125 μm) with 64% transmission were pretreatred with uranyl acetate (0.83% w/v) in ethanol (50% v/v) in the dark, for 10 mins. The grids were washed in 6 changes of deionised water then placed in Reynold's lead citrate for 60 secs. The grids were washed in 6 changes of deionised water and dried on filter paper. Sections (approximately 800 Å in dia.) of the Araldite-embedded cell pellets were placed on the pretreated grids. The sections were observed in a Phil ips 301 G Electron Microscope which had been standardized for 4% error.

5. COLLECTION OF MONONUCLEAR CELLS

5.1. Blood Samples (U. Schneider et al., 1975)

Blood samples were collected from the umbilical cords of full-term pregnancies (HUCB), or the peripheral blood of human subjects of varying ages (HPB). Blood samples were added to heparin (20 i.u./ml). The hematocrit of HUCB samples was monitored to 40% (v/v) by the addition of heparinized RPMI-1640 (20 i.u/ml). Mononuclear cells were prepared by Ficoll-hypaque density gradient centrifugation, from HUCB and HPB samples.

5.2. Preparation of Single Cell Suspensions

Mice (Balb/c) of varying ages were anaethesized with diethyl-ether for 20 mins. For the removal of livers or spleens, mice were first dipped in methanol to clean the skin of the animals. The skin just below the thoracic cavity was lifted with a pair of forceps and an incision made across the left side of the animal. Another incision was then made in a corresponding region of the peritoneal cavity. Spleens or livers were carefully removed from the abdominal cavity, and placed in CM (6.0 mls). The bulk of the organ was gripped with clean forceps and the cells gently teased out of their cavities. Single cell suspensions were obtained by passing the suspensions thrice through each of 4 hypodermic needles, fitted with syringes (10 ml capacity).

Hypodermic Needles

10g x 1½" ----- 1

21g x 1½" ----- 2

23g x 1" ----- 3

25g x 5/8" ----- 4

The needles were used in chronological order as indicated. The suspensions were left at room temperature for 1 min, to allow any small pieces of tissue to settle from the cell suspensions. Mononuclear cells were collected by Ficoll-hypaque density gradient centrifugation.

5.3. Ficoll-hypaque Density Gradient Centrifugation

(A. Böyum, 1968)

Equal volumes of samples were carefully layered over Ficoll-hypaque solution and the gradients centrifuged at 350 xg for 30 mins.

Ficoll-hypaque Solution

Ficoll 400 (9% w/v)	70.59% (v/v)
Sodium hypaque (33.9% w/v)	29.41% (v/v)

density, 1.078 gm/ml

The solution was sterilized by Millipore filtration (0.45 μm in dia. pore size)

The opalescent band of mononuclear cells between the medium and the Ficoll-hypaque solution was removed and the cells collected by centrifugation, washed in three separate changes of RPMI-1640 with antibiotics and in one

change of CM. The density of the mononuclear cell suspension was determined with white cell counting fluid, and the %age viability determined by the Trypan blue exclusion test.

Under these conditions, >90% viable, mononuclear cells were obtained from the samples under test. Fig. 1. exemplifies the morphology of the cells, which were obtained from human blood samples.

5.3.1. Determination of Cell Density

Equal volumes of white cell counting fluid and cell suspensions were mixed and left at room temperature for 10 mins.

White Cell Counting Fluid

Gentian violet (0.1% w/v) in glacial acetic acid (2%).

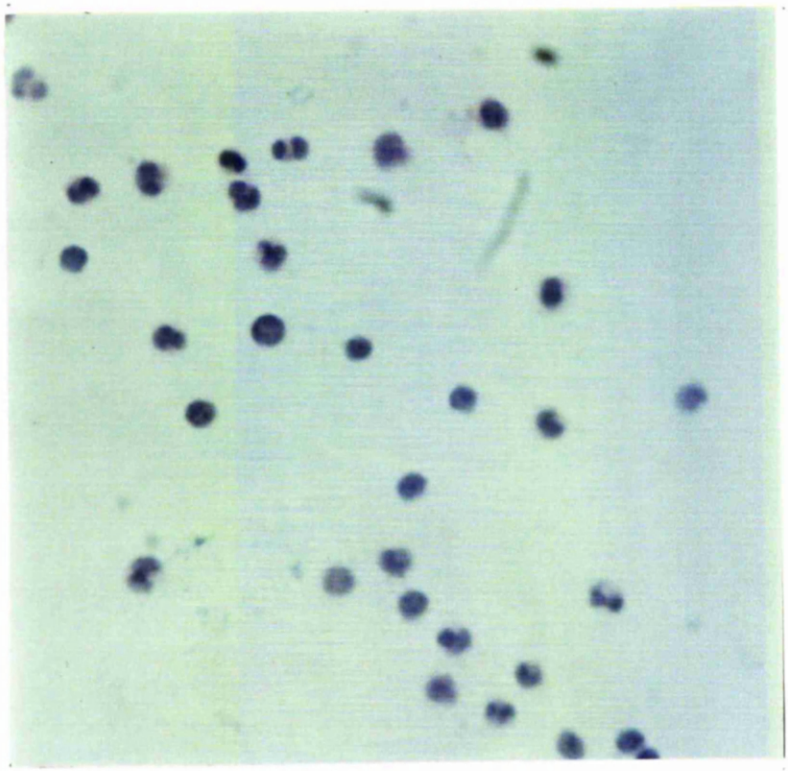
The unlysed cells were counted in a Neubauer hemocytometer counting chamber.

6. ESTABLISHMENT OF EBV-TRANSFORMED CELLS

6.1. Infection of Mononuclear Cells (adapted from T. Katsuki et al., 1975)

All steps were carried out under sterile conditions. Mononuclear cells were obtained from human umbilical cord blood samples by Ficoll-hypaque density gradient centrifugation. After washing the cells in CM, the

Fig. 1. Human mononuclear cells, prepared from human umbilical cord blood, by Ficoll-hypaque density gradient centrifugation and stained by Giemsa's staining technique. (Magnification X40)



viability of the cell suspensions were determined by the Trypan blue exclusion test and the densities brought to 2.4×10^6 cells/ml with freshly prepared CM.

EBV B958 or EBV QIMR-WIL was diluted in CM and equal volumes of virus and cell suspensions were mixed. Equal volumes of cell suspensions (without added virus) and CM, were also mixed. The suspensions were incubated at 37°C for 90 mins, shaking the culture flasks every 30 mins to facilitate adsorption and penetration of the cells. The cells were collected by centrifugation, suspended in CM to their original density and aliquots (1.0 ml) placed in screw-capped culture tubes (10.0 ml capacity). The tubes were incubated at 37°C and observed for the appearance of the transformed state.

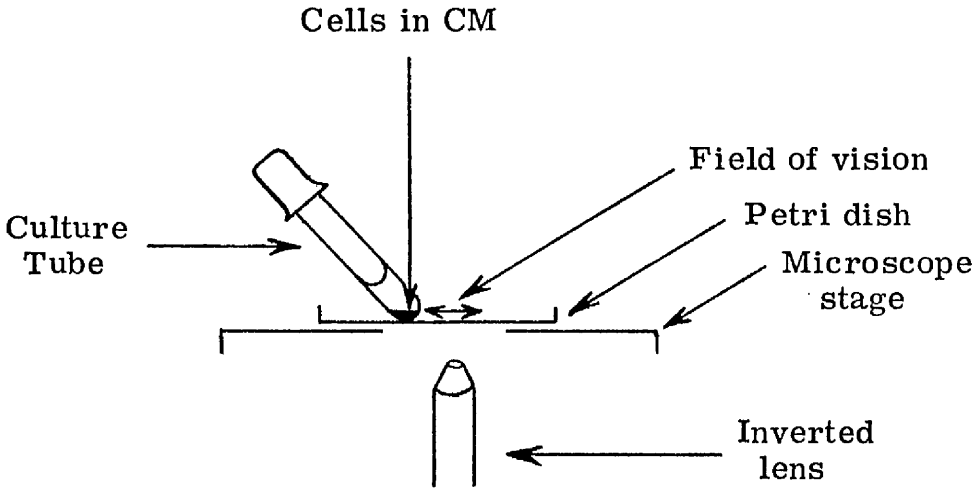
6.2. Determination of the Transformed State :

Establishment

Every 2 days after infection, each tube was examined for the evidence of the transformed state. This was done by placing the tube on the inside of a small (50 mm) petri dish which had been placed on the stage of a Leitz inverted microscope. The area of the tube which contained the cells was then gradually brought within the field of vision. Fig. 2 illustrates the technique.

The initiation of the transformed state was characterized by the appearance of EBV-transformed foci of cells at the bottom of the tubes, which was eventually coupled with acidity of the medium.

Fig. 2 Observation of EBV-infected cells by Leitz Inverted Microscopy



Approximately 2 weeks after the transformed state had been established, cell suspensions were transferred to tissue culture flasks (25 cm^2 or 75 cm^2). Cell suspensions were also maintained as duplicate cultures in tissue culture tubes. I found that it was adequate to replace 3 parts of the cell suspensions in the tubes, by freshly prepared CM every 7 days. On the other hand, 3 parts of the cell suspensions in the tissue culture flasks were replaced by CM every 3 or 4 days, for the efficient maintenance of cultures. After 3-4 months in culture (relative to the time of establishment of the transformed state), the EBV-transformed cells (lymphoblastoid cell lines) were considered fully established. Under these conditions, some infected cells failed to survive the continual passages.

EBV B958 or EBV QIMR-WIL transformed cells grew as clumps of cells in suspension (Fig. 3). The size of the clumps varied from small to large and was independent of the viral strain. Fig. 4. illustrates the uninfected, untransformed mononuclear cells in suspension, which had been obtained from HUCB.

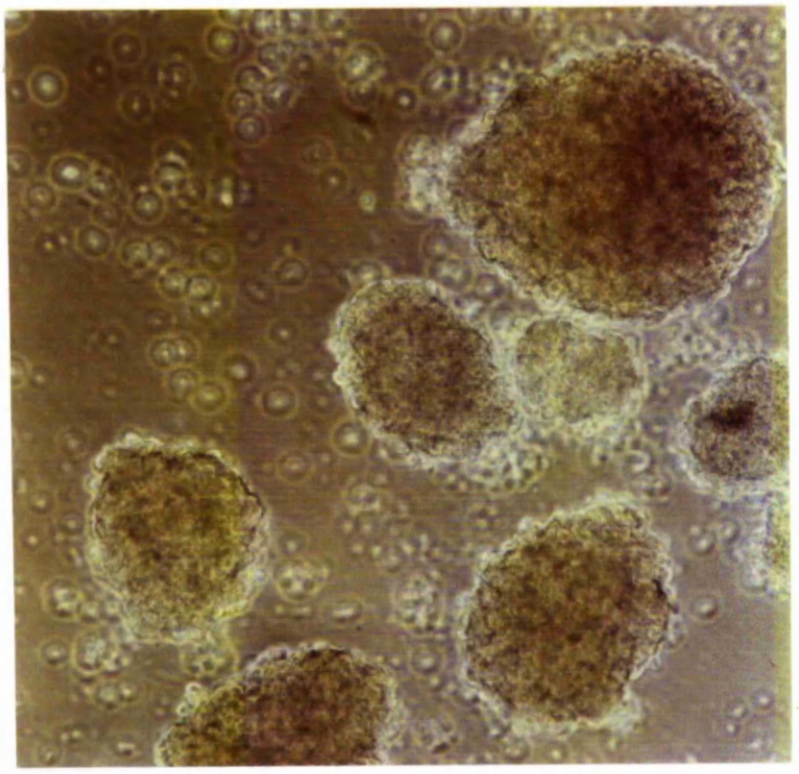
7. MAINTENANCE OF EBV-INFECTED CELL LINES

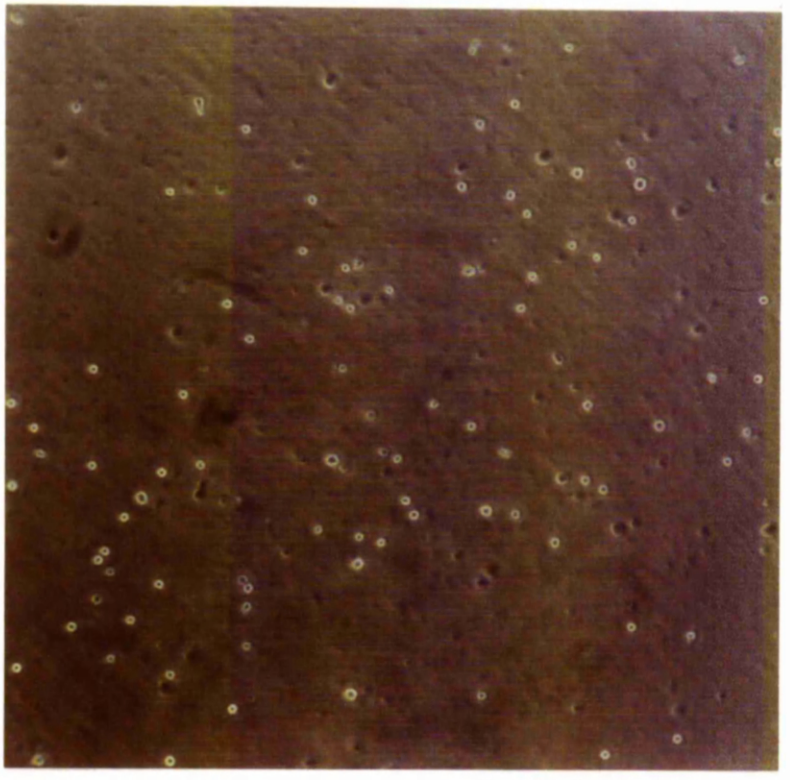
7.1. Cultural Conditions

All cell lines were maintained at 37°C in an atmosphere of 5% CO_2, and 95% relative humidity, when in culture.

Fig.3. EBV-transformed mononuclear cells in suspension.
(Magnification X10)

Fig.4. Uninfected, untransformed human mononuclear cells in suspension.
(Magnification X10).





7.2. Maintenance of Cell Stocks

Stocks of established lymphoblastoid cells were stored in liquid nitrogen. This was done by determining the viability of each LCL from 2-4 day-old cultures by Trypan blue exclusion test. Cultures which were 80-90% viable were stored in 1-2 mls freezing medium ($3-5 \times 10^6$ cells/ml).

Freezing Medium

Inactivated FCS	20% (by volume)
RPMI-1640	70% (by volume)
glycerol	10% (by volume)

The cell suspensions were stored at -70°C for 24 hrs, and then transferred to liquid nitrogen.

7.3. Rescue of Cell Stocks

Stocks of cell suspensions were brought from liquid nitrogen at room temperature and resuspended into equal volumes of preincubated (37°C) CM with 20% FCS. One half of the culture medium was replaced by CM with 20% FCS every 2-3 days, until the metabolic processes of the cells had been restored.

8. CONTAMINATION CHECKS

8.1. Pleuropneumonia-like Organisms (PPOs)

8.1.1. Monolayer Cultures

Cells were grown on coverslips at 37°C for 3 days.

All other steps were carried out at room temperature. The cells were left in sodium citrate solution (0.64% w/v, in distilled water) for 10 mins. The cells were washed in two separate changes of Carnoys solution for 15 mins each wash.

Carnoys Solution

Glacial acetic acid	25% (v/v)
Absolute alcohol	75% (v/v)

Orcein staining solution was added to the cells. The cells were left for 20 mins and then washed in three changes of distilled water.

Orcein Staining Solution

Orcein stain	2% (by volume)
Glacial acetic acid	60% (by volume)
Distilled water	40% (by volume)

The cells were mounted in euperol mounting medium on glass slides and observed with a Leitz Ordinary Light Microscope (X100 lens) for the appearance or absence of densely stained PPOs in the smears.

8.1.2. Suspension Cultures (Gibco-bio-Cult, Europe Ltd.)

Mycoplasma broths (LMV) were inoculated with 75 mls cell suspension and incubated at 37°C.

LMV Broths

	g/l
Gibco Mycoplasma broth base	21.00
Dextrose	10.00
Gibco yeast extract	25.00
Arginine HCl	10.00
Thallous acetate	0.50
Penicillin "G"	0.12
Phenol Red	0.02
Medium 199 Nucleotides (X100)	(1.00%)

pH 7.35 - 7.45, Osmolarity 270-500

Gibco Mycoplasma Broth Base

	g/l
Beef heart infusion	2.00
Peptone 220 (yeast caesin polypeptone)	10.00
Beef extract	3.00
Sodium chloride	5.00

After 3, 10 and 17 days, Mycoplasma agar was inoculated with the broth cultures, by indentation of the surfaces of the agar. The cultures were incubated at 37°C in anaerobic conditions with an atmosphere of 5% CO_2/95% nitrogen.

Mycoplasma Agar

*Gibco Mycoplasma base agar	34 g/l)
Gibco gamma globulin-free horse serum	20% (by volume)
Yeast extract (25%)	0.4% (by volume)
Penicillin "G" (10 i.u./ml)	0.2% (by volume)
Thallium acetate (5%)	1.0% (by volume)

*Gibco Mycoplasma agar base was sterilized by autoclave (15 lbs/sq in for 20 mins, at 121°C). The solution was then cooled to 48°C, added to the other components of the medium and the plates poured.

Mycoplasma agar plates were examined by ordinary light microscopy at 7 and 21 days after incubation, for the presence (typical "fried-egg" appearance of colonies) or absence of Mycoplasma.

8.2. Bacterial

Blood agar plates were prepared according to the following formulation :-

Bacterial Agar/10 plates

50 ml Blood agar "Difco Bacto"

5 ml Horse blood.

Concentrated cell suspensions were spread on the blood agar plates and the plates incubated at 37°C in a humid chamber. If positive, colonies developed 2-5 days.

9. IMMUNOFLUORESCENCE OF ANTIGEN-ANTIBODY POSITIVE CELLS

9.1. General

All microscope glass slides (76 x 26 mm) and glass coverslips (13 mm in dia.) were cleaned in methanol before use. The smears were never allowed to dry throughout the experimental procedures.

Viable cells were used for the detection of sIg^+ cells. Fixed cells were used for the detection of intracellular fluorescence-positive cells.

Suitable smears (see Table 2) were included in each test as controls for the experiments.

The mounting medium which was used throughout consisted of glycerol/PBS (50% v/v).

All smears were observed by u.v. illumination with a Leitz Ortholux u.v. microscope. At least 500 cells were counted on each smear to determine the %age IF^+ cells. Fixed smears were not stored for longer than 48 hrs before observation. Unfixed smears were observed within 5 hrs of preparation.

9.2. Evan's Blue Counterstaining

All steps were carried out at room temperature. The smears were washed in three changes of PBS-A and in one change of distilled water. Evan's blue staining solution (0.005% w/v) was added, and the smears left for 10 mins.

Table 2 . Characteristics of Cell Lines (Controls)

Designation	IF Method	*IF Reactivity	Reference
Raji	ACIF	$EBNA^+$	B. Reedman et al., 1973
		$(EA/VCA)^-$	B. Hampar et al., 1974
BJAB	ACIF	$EBNA^-$	B. Reedman et al., 1973
P_3HR-1	DR IF	$(EA/VCA)^+$	G. Kleins' personal communication.
MPC-315	IND IF	sIgA $(\lambda_2)^+$	Laboratory clones
MPC-11	IND IF	sIgG $(k)^+$	" "
Daudi	DIR IF	s/c IgM	T. Kishimoto et al., 1978;
		$(\mu/k)^+$	E. Klein et al., 1968
RPMI-1788	DIR IF	s/c IgM	T. Kishimoto et al., 1978
		$(\mu/\lambda)^+$	
Bec-11	DIR IF	s/c IgG	Laboratory determined
		$(\gamma/k)^+$	

Abbreviations : ACIF = Anti-complementary immunofluorescence
IF = Immunofluorescence.
c, s = cytoplasmic, surface.
* +, - = Positive, Negative
DR, IND = Direct, Indirect.

Evan's Blue Staining Solution

Evan's blue (dry powder) was dissolved in distilled water and stored as a stock, at room temperature as a 0.01% (w/v) solution.

The smears were washed in distilled water, in three successive changes of PBS, mounted, and observed by u.v. illumination.

9.3. Direct Immunofluorescence

9.3.1. Detection of Surface Immunoglobulins (J. Nadkarni et al., 1969)

Cells were washed in two changes of PBS-A, collected by centrifugation and resuspended in PBS-A (2×10^6 cells/ml). Cells (2×10^5/100 μl) were added to equal volumes of FITC-conjugated RAH antisera (Behring), which had been raised against IgM (μ), IgG (γ) heavy, Ig (k), Ig (λ) light chains, and diluted 1 in 10 in PBS-A. The suspensions were mixed and incubated at 37°C for 30 mins. Cells were collected by centrifugation, washed in 2 successive changes of PBS-A and suspended in mounting medium (20 μl/2×10^5 cells). Aliquots (10 μl) were placed on microscope glass slides, coverslips added and the smears immediately observed by u.v. illumination for the fluorescence of Ig molecules on the surfaces of cells.

9.3.2. Detection of Cytoplasmic Immunoglobulins (Y. Hinuma et al., 1967)

Smears were prepared from 2 day-old cultures of LCLs by cytocentrifugation and fixed in acetone at room temperature for 10 mins. The smears were washed in PBS-A. 100 µl FITC-conjugated RAH antisera (Behring) which had been raised against IgM (µ), IgG (γ) heavy; and Ig (k), Ig (λ) light chain types, and diluted 1 in 10 in PBS-A were added. The smears were incubated at 37°C for 30 mins, counterstained in Evan's blue staining solution, mounted and observed by u.v. illumination for the fluorescence of Ig molecules in the cytoplasms of cIg^+ cells.

9.3.3. Detection of Early and Virus Capsid Antigens (G. Klein's personal communication).

Smears were prepared from 2 day-old cultures of LCLs by cytocentrifugation and fixed in acetone at -20°C for 10 mins. The smears were washed in PBS-A, FITC-conjugated VCA^+ (F_1-Katana) or $(EA/VCA)^+$ (F_3-Esther) antisera which were diluted 1 in 40 in PBS-A were added (100 µl). The smears were left at room temperature for 1 hr, counterstained in Evan's blue staining solution, mounted and observed by u.v. illumination for the intracellular fluorescence of VCA and/or EA positive cells.

9.4. Anticomplementary Immunofluorescence (B. Reedman et al., 1973)

Smears were prepared by cytocentrifugation, fixed at

-20°C for 15 mins and washed in PBS-A.

ACIF Fixative

Acetone	2 parts
Methanol	1 part

100 µl $EBNA^+$ (Walter Owino) or $EBNA^-$ (Ingela) human antiserum (a/s) which had been diluted 1 in 10 in PBS-A, was added. The smears were incubated at 37°C for 15 mins, washed in three separate changes of PBS-A and 100 µl complement (C', Eva Klein) which had been diluted 1 in 10 in PBS-A, added. The smears were incubated at 37°C for 15 mins and washed in three separate changes of PBS-A. 100 µl FITC-conjugated anti-β_{1c} component of complement (FITC-β_{1c}, Dr. George Klein), which had been diluted 1 in 20 in PBS-A, was added. The smears were left at room temperature for 30 mins, counter-stained in Evan's blue solution, mounted and observed by u.v. illumination for nuclear fluorescence of EBNA.

The following tests controlled the ACIF test

BJAB, Raji, HUCLs : $EBNA^+$ or $EBNA^-$ a/s; C'; FITC-anti-β_{1c}

BJAB, Raji, HUCLs : $EBNA^+$ or $EBNA^-$ a/s; C'; PBS-A

BJAB, Raji, HUCLs : $EBNA^+$ or $EBNA^-$ a/s; PBS-A; FITC-anti-β_{1c}

BJAB, Raji, HUCLs : PBS-A ; C' ; FITC-anti-β_{1c}

Nuclear staining for EBNA was never observed when BJAB or HUCLs were tested against $EBNA^+$ antiserum. On the other hand, Raji always displayed nuclear fluorescence for EBNA when Walter Owino, but not when Ingela antiserum was used in the ACIF test.

9.5. Indirect Immunofluorescence (D.M. Weir, 1978)

Mononuclear cell suspensions were prepared from single cell suspensions by Ficoll-hypaque density gradient centrifugation. The cells were washed in two successive changes of PBS-A. The densities of the mononuclear cell suspensions were determined with white cell counting fluid, and the %age viability of each suspension determined by the Trypan blue exclusion test. Cells were collected by centrifugation and resuspended in PBS-A (2×10^6 cells/ml).

100 μl, containing 0.6 mg Rabbit anti-mouse (RAM) antiserum (Table 3) which had been raised against Ig (k) or Ig (λ_2) in PBS-A, was added to 2×10^5 cells (in 100 μl PBS-A). The cell suspensions were mixed and incubated at $37^{\circ}C$ for 30 mins - in some cases, cells were incubated at $4^{\circ}C$. Cells were collected by centrifugation at 200 xg for 10 mins, washed in two changes, and resuspended in, 100 μl PBS-A. 100 μl FITC-conjugated GAR IgG, containing 0.7 mg protein (FITC-GAR IgG) was added, the cell suspensions mixed, and incubated at $37^{\circ}C$ (or $4^{\circ}C$). Cells were collected by centrifugation, washed in two changes of PBS-A and collected by centrifugation. Mounting medium was added (20 μl/2×10^5 cells), aliquots (10 μl) placed on microscope glass slides, coverslips added, and the smears immediately observed by u.v. illumination for the fluorescence of surface immunoglobulin positive cells.

The following tests controlled the Indirect IF :-

MPC-315, MPC-11 : RAM Ig (k) or Ig (λ_2) ; FITC-GAR IgG.

MPC-315, MPC-11 : PBS-A ; FITC-GAR IgG.

MPC-315, MPC-11 : RAM Ig (k) or Ig (λ_2) ; PBS-A

MPC-315, MPC-11 : NRS (1:10 in PBS-A) ; FITC-GAR IgG

MPC-315, MPC-11 : NRS (1:10 in PBS-A) ; PBS-A

Abbreviation: NRS = Normal Rabbit Serum.

There was no statistically significant difference observed between the %age sIg^+ cells, whether the experiment was conducted at 37°C or 4°C. sIg^+ cells were never observed when NRS and FITC-GAR IgG were added to MPC-315 or MPC-11. sIg^+ cells were never observed when MPC-315 were tested against RAM Ig (k) or when MPC-11 were tested against RAM Ig (λ_2) with FITC-GAR IgG. sIg^+ cells (30-40%) were observed when MPC-315 were tested against RAM Ig (λ_2) and when MPC-11 were tested against RAM Ig (k) with FITC-GAR IgG. RAM Ig (λ_2) was specific for λ^+ while RAM Ig (k) was specific for k^+ cells.

10. Acquisition of Laboratory Prepared Antibodies

Table 3 displays a summary of the techniques, and antigens which were employed for the preparation of antibodies.

10.1. Boric Acid Precipitation of Euglobulins

Serum samples containing the myeloma proteins were centrifuged at 10,000 xg to remove insoluble material. The centrifuged samples were then added in a dropwise manner, while mixing, to solutions of 2% (w/v) boric acid (twenty

Table 3. Acquisition and Purification of Antibody Preparations

Antibody	From Antisera	Against Antigen	Selected On Immunoadsorbent
RAM IgA (λ_2)	Bo/Ac DNP-Lys(SR) G-100	MPC-315 (IgA (λ_2))	IgA (λ_2)
RAM IgG_{2b}(k)	$(NH_4)_2SO_4$ A-50	MPC-11 (IgG_{2b}(k))	IgG_{2b}(k)
GAR IgG	G-200	Rb IgG	Nordic IgG
RAH IgM	Bo/Ac G-200	IgM k(7A)	IgM (λ)

Abbreviations : G-200, A-50, G-100 = Ion Exchangers

$(NH_4)_2SO_4$ = Ammonium sulphate

Bo/Ac = Boric Acid

DNP-Lys(SR) = DNP-lysine (Sepharose)

Note : All antibody preparations were determined to be pure by immunoelectrophoretic techniques. There was no cross-reactivity observed between RAM IgA (λ_2) and RAM IgG_{2b}(k).

times the volume of the serum samples). After standing at room temperature for 2 hrs, the precipitates were collected by centrifugation at 10,000 xg for 30 mins. The precipitates were then dissolved and dialysed against G-200, or DNP-lys (Sepharose) buffers.

10.2. Ammonium Sulphate Precipitation

The serum sample containing the immunoglobulin protein was centrifuged at 10,000 xg for 15 mins to remove insoluble material. The serum sample was then diluted with equal volume of PBS-A, and saturated ammonium sulphate (53.6% w/v) added in a dropwise manner, while mixing, until 40% saturation was attained. After leaving at room temperature for 2hrs, the precipitate was collected by centrifugation at 10,000 xg for 30 mins. The precipitate was washed once with 40% saturated ammonium sulphate and then dissolved in a suitable volume of PBS-A. The solution of antiserum was then dialysed extensively (48 hrs) against A-50 column buffer.

10.3. Sephadex G-200 Column Chromatography

A column (2.5 cm in dia. x 100 cm in length in a bed volume of 500 ml) was packed with Sephadex G-200 which had been equilibrated in column buffer and degassed.

G-200 Column Buffer

0.1 M Tris-HCl

0.2 M NaCl

pH 8.0

The dialysed sample of antiserum was centrifuged at 30,000 xg for 30 mins to remove insoluble material and the sample carefully added to the sample bed. This was done by removing any excess column buffer which was at the top of the column bed and the sample slowly added to the column bed. The column was then connected by an air-tight system to a reservoir of column buffer. The reservoir of column buffer was at a height above the level of the column head, to enable the efficient passage of the reservoir buffer, and maintenance of the flow rate. The flow rate (10-15 mls/hr) was achieved by the adjustment of the hydrostatic pressure head. Fractions (2-5 mls) were collected, the absorbance (A_{280}) determined, the peak fractions containing the antigen pooled, and concentrated by Amicon filtration.

10.4. Sephadex A-50 Column Chromatography

A column (2.5 cm in dia. x 100 cm in length in a bed volume of 500 ml) was packed with Sephadex A-50 which had been equilibrated with column buffer and degassed.

A-50 Column Buffer

1 M KH_2PO_4

1 M K_2HPO_4

pH 8.0

A 0.01 M solution was prepared for use.

The dialysed sample of antiserum was centrifuged and applied to the column bed as detailed in 10.3. The protein fractions were eluted with a gradient of column buffer, 0.01 M - 0.30 M. The absorbance (A_{280}) of the fractions

were determined, the peak fractions pooled and concentrated by Amicon filtration.

10.5. DNP-Lysine (Sepharose) Column Chromatography

A column (1.0 cm in dia. x 20 cm in length, in a bed volume of 1 ml/ml sample) was packed with DNP-lysine (Sepharose), DNP-Lys(SR), which had been washed with 5 volumes acetic acid (25%) and 10 volumes column buffer.

DNP-Lys(SR) Column Buffer

0.2 M Tric-HCl, pH 8.0

The dialysed serum sample was filtered by Millipore filtration and equal volume of column buffer added. The sample was added to the DNP-lys(SR) column. Two separate volumes of column buffer/dithiothreitol (DTT) was added, the flow stopped, and the column left at room temperature for 30 mins after each addition. The column was washed with two changes of 10 mM iodoacetic acid (IAA). 0.2 volume of column buffer/0.01 M DNP-aminocaprioic acid (APA) was added and a reservoir of column buffer applied as detailed in 10.3. Fractions were collected and the absorbance determined as follows :

A_{280}(Protein); A_{360}(DNP-APA) : A_{280}-0.385(A_{360}) = A_{280}

The peak fractions were pooled, dialysed against column buffer, partially reduced, and alkylated.

10.5.1. Partial Reduction and Alkylation

50 mM DTT, and 20 µl phenol red was added to the dialysed samples and the mixture incubated at 37°C for 30 mins. 150 mM IAA was added, while stirring, 0.1 volume 1 N NaOH (pH 8-8.4) added and the mixture left at room temperature for 15 mins. The samples were then dialysed against G-100 column buffer for 48 hrs.

10.6. Sephadex G-100 Column Chromatography.

A column (1.0 cm in dia. x 20 cm in length in a bed volume of 1 ml/ml sample) was packed with Sephadex G-100 which had been equilibrated with column buffer and degassed.

G-100 Column Buffer

6 M Urea-Formate, pH 8.0

The dialysed samples were centrifuged and applied to the column beds as detailed in 10.3. Fractions were collected, the absorbance (A_{280}) determined, the peak fractions pooled, and concentrated by Amicon filtration.

10.7. Acquisition of Rabit Antisera

An equal volume of immunoglobulin preparation (0.5 - 1.0 ml) was mixed with complete Freund's adjuvant. After emulsification of the mixture by sonication, it was administered to the rabbits in the form of two primary injections, intravenously (0.2 - 1.0 mg per injection). Within the fourth week after the second primary injection, booster injections were administered at various sites of the animal,

subcutaneously (0.2 mg protein per injection). Within the sixth week after the final booster injection, the animals were bled from the ear veins. The rabbits were repeatedly bled at 1-2 week intervals, 20-50 mls blood being taken at each bleeding. The blood samples were incubated at 37°C for 30 mins and then at 4°C for 20 hrs to enable clotting to take place. The serum samples were filtered and stored at -20°C until ready for use.

10.8. Sepharose Affinity Chromatography

Sepharose 4B was washed extensively in distilled water and then activated in a fume cupboard with cyanogen bromide (CNBr), using; 100 mg CNBr/ml sepharose, but 25% (v/v) sepharose. The pH of the sepharose was then monitored with 5 N sodium hydroxide to 11.0 - 11.5, while mixing. The sepharose was then immediately washed in, and resuspended in, ice-cold 0.1 M $NaHCO_3$, using five times the volume of the sepharose.

The sample to be coupled (1-10 mg Ig/ml sepharose) was then added and the reaction allowed to continue at 4°C for 16 hr, while mixing. Eluates were collected and the sepharose washed with 0.1 M $NaHCO_3$. The absorbance (A_{280}) of the eluates was determined and the total uncoupled immunoglobulin determined. The amount of coupled Ig was calculated according to the following formula :-

Coupled Ig = (Total Ig) - (Uncoupled Ig).

The sepharose was then reacted at 4°C for 16 hr with

a molar equivalent (relative to the CNBr) of glycine to quench any remaining active sites, washed in, and resuspended in, PBS-A containing 0.05% sodium azide as the preservative. The sepharose was stored at $4^{\circ}C$.

10.9. Purification of Antibody Preparations

The sepharose-bound Ig was loaded into a small disposable syringe which served as a column. The column was prepared by cutting a Millipore glass fibre filter to the appropriate size and placing it at the bottom of the syringe. The antiserum to be purified was applied to, and allowed to pass through the column, at room temperature. Fractions were collected, the column washed once with PBS-A (v/v antiserum), and twice with at least five times the volume of antiserum, with PBS-A. The absorbance of the fractions was noted (A_{280}) and the washings suspended when A_{280} began to decrease to near zero. The bound antibodies were eluted with one of the following solutions :-

0.1 M Acetic Acid

1.0 M Propionic Acid

0.1 M Glycine HCl, pH 2.8.

The eluates were collected, the elution buffer neutralized with a suitable volume of 1 M Tris, and the absorbance (A_{280}) noted until it began to decrease significantly to near zero. The peak fractions were pooled, dialysed against PBS-A, concentrated, and stored in aliquots at $-20^{\circ}C$.

11. FITC-Conjugation of GAR IgG (D.M. Weir, 1978)

GAR IgG was centrifuged at 30,000 xg for 30 mins to remove insoluble material and chilled. 0.5 M sodium bicarbonate buffer (10% v/v) was added.

Sodium Bicarbonate Buffer

$NaHCO_3$	3.7%	w/v
Na_2CO_3	0.6%	w/v
		pH 9.0

Fluorescein isothiocyanate (FITC) was added (50 μg/mg Ig protein), while mixing, and the reaction allowed to proceed for a further 6 hrs at room temperature. The conjugated antibody preparation was then dialysed against PBS-A for 48 hrs, centrifuged at 30,000 xg for 1 hr to remove insoluble material and stored in aliquots at -20°C.

12. ANALYSIS OF LABELLED IMMUNOGLOBULIN PROTEINS

12.1. Biosynthetic Labelling of Lymphoblastoid Cells

(Adapted from an unpublished manuscript of Dr. T. Mossman)

Cells from 2 day-old cultures of cell lines which had been maintained in culture for 18-20 months after the establishment of the transformed state were washed in two changes, and suspended in, labelling medium.

Labelling Medium

RPMI-1640 without L-leucine

or L-methionine

Lyophilized, labelled ^{3}H-leucine, or ^{35}S-methionine

(100 μCi) was dissolved in labelling medium (100 μl) and added to 4 x 10^6 cells in 100 μl labelling medium. The suspensions were incubated at 37°C after mixing, and mixed every 15 mins after incubation, to disrupt cell clumps.

12.2. Collection of Intracellular Proteins

All steps were carried out in an ice bath. Samples were removed from biosynthetically labelled cell suspensions after 30 mins incubation, and added to ice-cold CM (20 parts to 1 part cell suspension). The cells were collected by centrifugation and the pellets completely disrupted. The cells were then resuspended in lysis buffer-1 ($4\frac{1}{2}$ times the original volume of the cell suspension).

Lysis Buffer-1

TKM (X10)	8 parts
Triton X-100 (10% v/v)	1 part

TKM (X10)

Tris-HCl, pH 8.2	100 mM
potassium chloride	100 mM
Magnesium chloride	5 mM

The cell suspensions were left for 15 mins, to facilitate cellular lysis and the cytoplasmic lysates collected by centrifugation at 400 xg for 15 mins. Lysis buffer II was added (1 part to 9 parts cytoplasmic lysate) and the cellular debris removed by centrifugation at 30,000 xg for 1 hr.

Lysis Buffer II

Sodium deoxycholate	10% (w/v)
SDS	5% (w/v)

12.3. Effect of Cell Density on the Level of Incorporation

Various cell densities (1-7 x 10^6) from 2 day-old cultures of QWELB78 which were >90% viable were labelled biosynthetically (12.1., Methods) with ^{3}H-leucine (100 μCi). Intracellular proteins were collected (12.2., Methods), immune precipitates obtained (12.6., Methods) and the %age incorporation determined for each cell density which was used in the test, by Liquid Scintillation Spectrometry (12.5., Methods). The data was represented graphically as illustrated in Fig. 6.

The %age incorporation was found to increase with the number of cells, up to and including a cell density of 5 x 10^6 cells. Similar results were obtained with other cell lines (B9ELB78 and QWIM77) which were substituted for QWELB78 in this test. 4 x 10^6 cells were used throughout the analysis of Ig proteins of 18-20 month-old cultures because this density from cultures which were >90% viable was most easily attained from 2 day-old cultures.

12.4. Collection of Secreted Proteins

(T. Mossman, unpublished Manuscript; J. Andersson et al., 1974)

After 2 (^{35}S-methionine label) or 4 (^{3}H-leucine label) hrs, samples (100 μl) were collected from biosynthetically labelled cells, and chilled. Ice-cold RPMI-1640 (350 μl) was added and supernatant solutions were collected by centrifugation at 400 xg for 15 mins.

3D-TKM buffer was added (one part to nine parts supernatant).

3D-TKM Buffer

Sodium deoxycholate (10% w/v)	1 part
Triton X-100 (10% v/v)	1 part
SDS (5% w/v)	1 part
TKM (X10)	1 part
Distilled water	6 parts

The cell-free extracts were centrifuged at 30,000 xg for 1 hr to further purify the extract.

12.5. Determination of Antibody Equivalence

Intracellularly labelled proteins were collected from biosynthetically labelled QWELB78 cells. Varying amounts (5-100 μg) of RAH IgM was added to constant volumes (50 μl) of samples in each of 4 sets of tubes. The samples were mixed and incubated at 37°C for 30 mins. Various quantities (10, 15, 25 or 50 μg) of GAR IgG were added to the 4 sets of tubes in turn, the suspensions mixed and incubated at 37°C for 30 mins. The samples were then left at room temperature for 30 mins and the reaction of immune precipitation prolonged at 4°C for 20 hrs. Precipitates were collected by centrifugation at 8,000 xg for 2 mins, washed in two changes, and dissolved in 50 μl 3D-TKM. The amount of radioactivity in each sample was determined by Liquid Scintillation Spectrometry.

Fig. 5 Determination of Antibody Equivalance at maximum level of Incorporation of ^{3}H-leucine into Intracellular IgM proteins of QWELB78

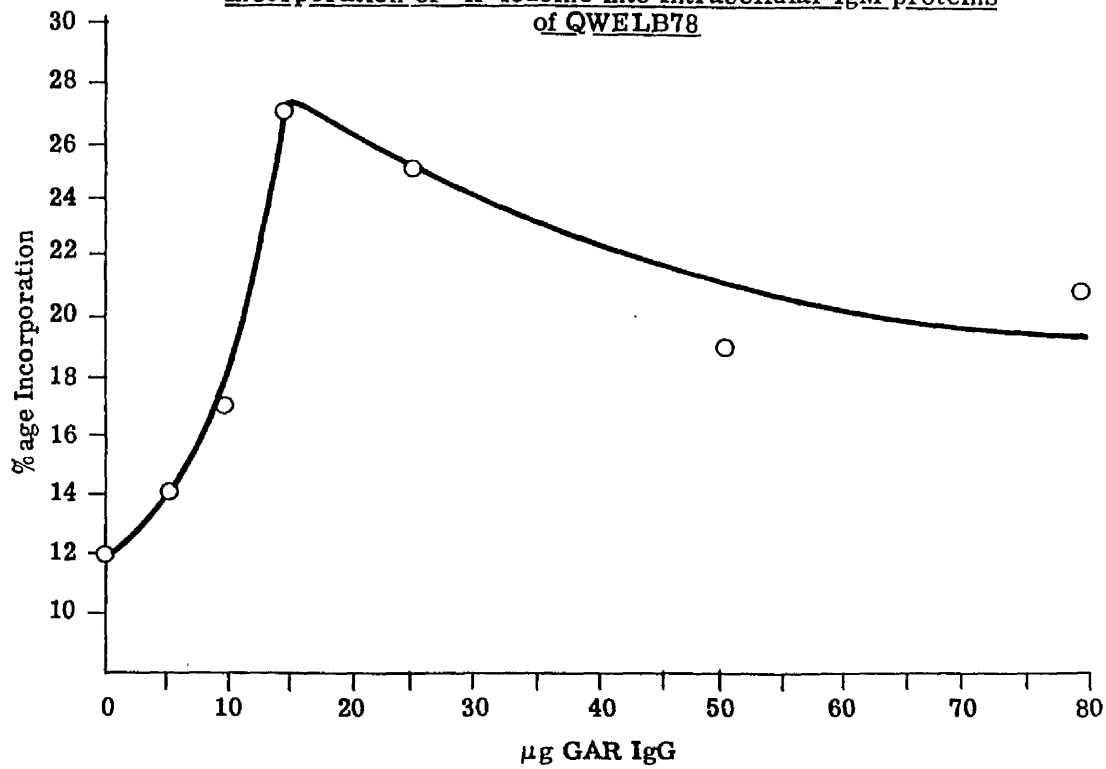
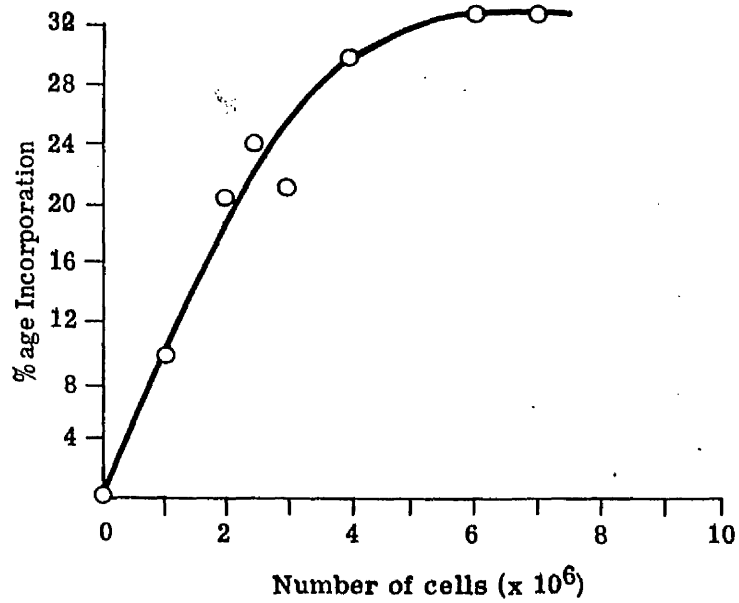


Fig. 6 Effect of Cell Density on the level of Incorporation of ^{3}H-leucine into Intracellular IgM proteins of QWELB78



12.6. Liquid Scintillation Spectrometry

Aliquots (10 µl) of samples were added to 5 µl CM. 1.0 ml 10% (w/v) TCA was added and the precipitates collected by centrifugation at 8,000 xg for 2 mins. The precipitates were dissolved in 100 µl Hyamine 10-X hydroxide and the mixture added to scintillation counting fluid (2.0 ml) as a 5% solution.

Hyamine 10-X Hydroxide

Scintillation grade with about 10% (w/v) in methanol

Scintillation Counting Fluid

PPO (0.4%) in toluene

The amount of radioactivity in each sample was determined in a Beckman LS 333 Scintillation Counter, with 85 and 90 % efficiency for β (^{3}H-) and γ (^{35}S-) radiation. The %age incorporation of labelled amino acids into immunoglobulin proteins was determined by the following formula :-

$$\%\text{age Incorporation} = \frac{\text{CPM of Sample}}{3.7 \times 10^{4}} \times 100$$

The maximum level of incorporation was achieved with 15 µg RAH IgM and 15 µg GAR IgG (Fig. 5). The maximal level of incorporation was found to be similar when RAH antisera which had been raised against γ, k or λ (Miles) antibodies were titrated against GAR IgG. The antibody equivalence was accepted to be achieved with 15 µg RAH antiserum and 15 µg GAR IgG.

12.7. Indirect Immunoprecipitation of Ig Proteins

RAH antisera which had been raised against μ, γ, k or λ antibodies (15 μg in 15 μl PBS-A) were added to aliquots (50 μl) of labelled intracellular or secreted protein samples. The samples were mixed and incubated at 37°C for 30 mins. 15 μg GAR IgG in 15 μl PBS-A was added, the samples mixed and incubated at 37°C for 30 mins. The samples were then left at room temperature for 30 mins to promote the development of immune precipitates, and the reaction prolonged at 4°C for 20 hrs. Precipitates were collected by centrifugation at 8,000 xg for 2 mins, and washed in two changes of 3D-TKM. Precipitates were collected by centrifugation at 8,000 xg for 2 mins and dissolved in 50 μl sample buffer for discontinuous SDS-polyacrylamide gel-electrophoresis. The radioactivity in 10 μl samples was determined by Liquid Scintillation Spectrometry and the exposure times for fluorographs determined.

13. DISCONTINUOUS SDS-POLYACRYLAMIDE GEL-ELECTROPHORESIS (U. Laemmli, 1970).

13.1. Stock Solutions

The following stock solutions were prepared as indicated and stored for not more than 1 month :-

Solution A	
Acrylamide	45% (w/v)
bis-Acrylamide	1.2% (w/v)
Solution B, pH 8.8	
Trizma Base	18.0% (w/v)
TEMED	0.13% (w/v)

Solution C, pH 6.8

Trizma Base	7.0 % (w/v)

Solution D

SDS	12% (w/v)

Glycine Reservoir Buffer, pH 8.8

Trizma Base	0.025 M
Glycine	0.192 M
SDS	0.10% (w/v)

Solutions A, B and C were stored at 4°C.

Solutions D and Reservoir buffer were stored at room temperature.

Sample Buffer

Solution C	1 part
Glycerol	1 part
SDS (10% w/v)	2 parts
DTT	100 mM
Distilled water containing bromophenol blue (0.01 mg/ml)	6 parts

The solution was stored at -20°C.

13.2. Preparation of Separating Gel

Two glass plates were aligned, but separated by plastic tubing (relative to the size of the gel), and secured in an upright position on a perspex stand. Separating gel (21 x 26 x 0.5 cm) containing 12.0 % acrylamide was prepared according to the following formulation :-

Separating Gel

33.3 mls	Solution A
30.0 mls	Solution B
1.0 ml	Solution C

The mixture was made up to 120 mls with distilled water, degassed, and ammonium persulphate (1 mg/ml) added to chemically induce the formation of free radicals. The mixture was immediately poured between the glass plates and left at room temperature for 1 hr to promote cross-linking and the subsequent polymerization of the acrylamide.

13.3. Preparation of Stacking Gel

The stacking gel which formed the discontinuous layer, contained 5% acrylamide and was prepared according to the following formulation :-

Stacking Gel

2.64 mls	Solution A
2.40 mls	Solution D
0.20 mls	Solution C
18.67 mls	Distilled water
24 µl	TEMED

A 26 place sample well template (made from Teflon) was inserted between the glass plates, above the top of the separating gel. The stacking gel solution was degassed, ammonium persulphate added, the mixture immediately poured and then left at room temperature for 20 mins to polymerize. When the gel had set, the sample well template was removed and the sample wells flushed with distilled water to remove

any undesirable fragment of stacking gel.

13.4. Electrophoresis of Samples

Samples (30 µl) of immunoprecipitates which had been dissolved in sample buffer, were reduced by heating at 100°C for 2 mins. Reservoir buffer was poured in the troughs of the perspex stand. The samples were then carefully loaded into the sample wells, underneath the Reservoir buffer. The samples were then electrophoresed at 12 mA/gel, with constant current, at 4°C for 20-26 hrs.

13.5. Fluorography of Slab Gels

(Adapted from W. Bonner et al., 1974)

The protein bands were fixed in the separating gel as follows :-

DMSO (250 ml)	20 mins
DMSO (250 ml)	20 mins
DMSO (250 ml)	40 mins

The gels were incubated at 30°C in a mixture of PPO/DMSO (20% w/v) for 45 mins with constant shaking, to facilitate complete impregnation of the gels. The excess PPO/DMSO was removed and the gel rinsed in, and washed in, running tap water for 1 hr, at room temperature. The gels were then dried on Whatmann chromatographic paper (3 mm) under pressure. The dried gels were placed between two glass plates (25 x 30 cm). Kodak X-Omat H film (24 x 30 cm) was inserted between the gel and the glass plate, ensuring that the film was in contact

with the dried gel. The gels were then placed in individual light proof vinyl film bags and left at -70°C for 21 (^{35}S-methionine) or 42 (^{3}H-leucine) days.

13.6. Development of Films

The films were processed at room temperature in the dark. Films were placed in DX-80 developer (Kodak) for 10 mins. The protein bands were fixed in FX-40 X-Ray liquid fixer (Kodak) for 10 mins. The films were washed in running tap water for 30 mins and air-dried at room temperature.

RESULTS AND DISCUSSIONS

Chapter 1

The Transforming Efficiencies of EBV Strains.

1.1. Establishment of Pairs of EBV-Transformed Cell Lines.

1.1.1. Introduction

Mononuclear cell populations were obtained from human umbilical cord blood samples (Methods, 5.1.) by Ficoll-hypaque density gradient centrifugation (Methods, 5.3.). Each sample of cell suspension was brought to 2.4×10^6 viable cells/ml with freshly prepared CM, and divided into three portions. EBV B958 or EBV QIMR-WIL stocks (Methods, 3.) were diluted in freshly prepared CM (4 parts to 1 part virus suspension). Each virus suspension in turn was mixed with equal volumes of one of three portions of cell suspensions. Equal volumes of the third portion of each cell suspension (without added virus) and CM were also mixed. The suspensions were incubated at 37°C, shaking the flasks every 30 mins, to facilitate adsorption and penetration of the cells.

Cells were collected from the infected and uninfected suspensions by centrifugation at 200 xg, after 90 mins incubation and suspended in CM to their original densities (1.2×10^6 cells/ml). Aliquots (1.0 ml, x3) from each infected and uninfected cell suspension were placed in screw-capped tissue culture tubes (10 ml capacity) and the suspensions incubated at 37°C. The suspensions were then observed daily with Leitz inverted microscope (Methods, 6.2) for the appearance of the transformed state.

1.1.2. Establishment of the Transformed State.

The transformed state was characterized by the appearance of transformed foci of cells when coupled with acidity of the medium. Transformed foci of cells were never observed among uninfected mononuclear cell populations. Approximately 3 days after infection and incubation, degeneration of the cultures began. While uninfected cultures continuously degenerated, the transformed state could be noted among pairs of EBV (B958) and EBV (QIMR-WIL) infected cells.

1.1.3. Designation of Pairs of Cell Lines.

The transformed state for 16 pairs of cell lines was noted. A pair of cell lines was designated following the acquisition of 2 cell lines which had been obtained from one sample of mononuclear cell preparation by infection with EBV B958 and EBV QIMR-WIL. Each pair was designated as follows :-

Virus strain	B9 or QW - -
Maternal donor e.g.	B9 or QW SD -
Year of establishment	B9 or QW SD 77 or 78

Samples of transformed cultures were transferred from tissue culture tubes to flasks (25 cm^2), approximately 14 days after the appearance of the transformed state, and subcultured every 3-4 days.

1.1.4. Discussion

Among the B9 infected cultures, the time lapse between infection and the establishment of the transformed state varied between 10 and 19 days, with an average of 12.8 ± 0.5 days; Table 1.1, columns 1 and 2. QW infected cultures on the other hand, remained in culture for nearly twice as long, before the transformed state was fully established. The time lapse for QW infected cells varied between 16.7 and 33.0, with an average of 24.2 ± 1.5 days; Table 1.1, columns 3 and 4. In most cases (Table 1.1), each time lapse determination was an average of 3 cultures.

When in suspension as single cells, B9 transformed cells were much larger than QW transformed cells. 1-2 days after subculture, some QW transformed cells adhered as monolayers to the culture flasks. However, this characteristic was not long lived. The "adherent" cells just as spontaneously as they adhered, resuspended themselves as clumps of cells before the next passage.

Table 1.1. Cell Lines and Time Lapses

B9 transformed		QW transformed	
Designation	TL (days)	Designation	TL (days)
B9SD77	12.3	QWSD77	17.3
B9FL77	13.0	QWFL77	22.0
B9MM77	10.0	QWMM77	20.0
B9AW77	13.0	QWAW77	23.7
B9RK77	17.0	QWRK77	31.0
B9EL77	19.0	QWEL77	33.0
B91M77	13.0	QW1M77	30.0
B9AB77	12.3	QWAB77	29.0
B9ELB78	10.0	QWELB78	26.0
B9E1F78	12.3	QWE1F78	16.7
B9SAM78	10.7	QWSAM78	16.7
B9BEB78	12.3	QWBEB78	26.0
B9LOC78	13.0	QWLOC78	22.0
B9MAC78	15.7	QWMAC78	28.0
B9E1B78	16.5	QWE1B78	27.0
B9MAC78	11.5	QWMAC78	18.3

Abbreviation : TL = Time Lapse

1.2. Kinetics of the Transformed State.

1.2.1. Introduction

Samples were collected from HUCB (Methods, 5.1). Mononuclear cells were collected by Ficoll-hypaque density gradient centrifugation (Methods, 5.3) and the viability and density of each cell suspension determined (Methods; 2.2, 5.3.1). Cell suspensions from 4 HUCB samples were infected with equal volumes of 4 separate suspensions of EBV B958 (10^{-1} dilution) as described in Methods, 6.1. Samples (1.0 ml x 60) from each of the 4 HUCB samples were incubated at $37^{o}C$. On days ; 4, 8 and 12 after infection, one half of the culture medium was replaced by freshly prepared CM without resuspending the cells. On days ; 17, 22 and 27, the cells in the suspensions were resuspended in the culture medium and one half replaced by freshly prepared CM. Cell suspensions (1.2×10^{6} cells/ml) without added virus were incubated as controls.

Samples (x3) were removed at various time intervals and the total number of viable cells determined by the Trypan blue exclusion test. The percentage blastoid cells in each sample was calculated by the following formula :-

$$\text{Percentage Blastoid cells} = \frac{\text{No. of Blastoid Cells}}{\text{No. of Viable Cells}} \times 100$$

Fig. 1D illustrates the mononuclear cells which were regarded as being blastoid in morphology.

Smears were prepared from each sample by cyto-centrifugation (Methods, 2.1) for the determination of percentage $(EBNA)^+$ cells (Methods, 9.4) for each sample. All smears were stored at $-70^{o}C$ until 15 and 30 days after infection, when half and all the samples had been collected. Fig. 1 represents the data collated from the 4 samples of HUCB which were treated to elucidate the kinetics of the transformed state.

1.2.2. Discussion

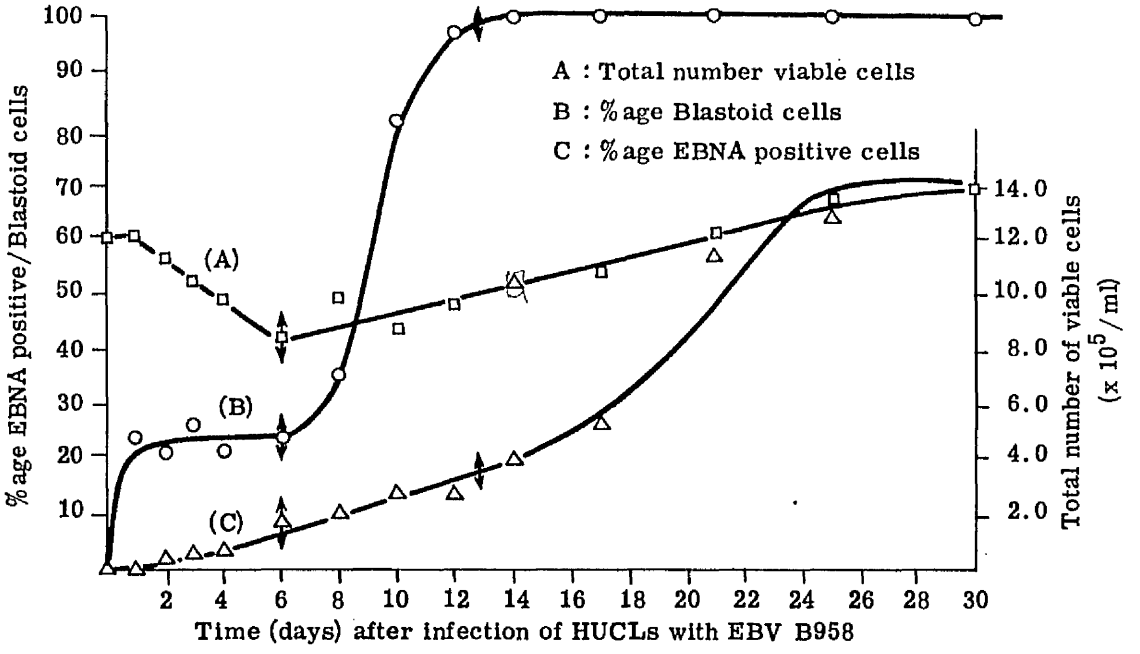
The total number of viable cells decreased from 1.1×10^6/ml on day 2 to 0.9×10^6/ml on day 6 (arrowed in Fig 1A) after infection. This decrease in the number of viable cells was due to the fact that uninfected cells in culture began to degenerate during this period (Results, 1.1.2). The decrease in viability of the samples coincided with a plateau of about 24% blastoid cells between 2 and 6 days, Fig. 1B. Between 6 and 13 days after infection (arrowed in Fig. 1B), the number of viable cells increased from 0.8×10^6/ml to 1.0×10^6/ml. This event coincided with a sharp increase in the percent blastoid cells as the B9 transformed cells began to proliferate logarithmically. The percentage blastoid cells increased from 23.5 to 99, 13 days after infection. A plateau of 100% blastoid cells was observed and maintained in the culture from 14 days after infection onwards.

These findings would suggest that, although EBV-transformed cells were in culture from as early as < 2 days (when $(EBNA)^+$ cells were detectable, Fig. 1C) after infection, a certain percentage of blastoid/B9-transformed cells has to be attained before the transformed state can become fully established. These conditions are applicable when a virus dilution of 10^{-1} (TD_{50}/ml = $10^{3.4}$) is used with cell density of 1.2×10^6/ml for the establishment of LCLs.

The appearance of the transformed state on day 2 was characterized by the detection of EBNA in 1.3% of the samples. The percent $(EBNA)^+$ cells increased from 1.3 on day 2 to 19.0 on day 14, when the transformed state was fully established. $(EBNA)^+$ cells increased from 19% on day 14 to 70% on day 27 after infection. The detection of blastogenesis occurred before the detection of EBNA in this system. 24 hrs after infection, 20% of the cells was blastoid, when EBNA was not detectable. Once the transformed state had been fully established, the number of $(EBNA)^+$ cells increased at a much faster rate (day 14 onwards) than prior to the establishment of the transformed state (0-14 days).

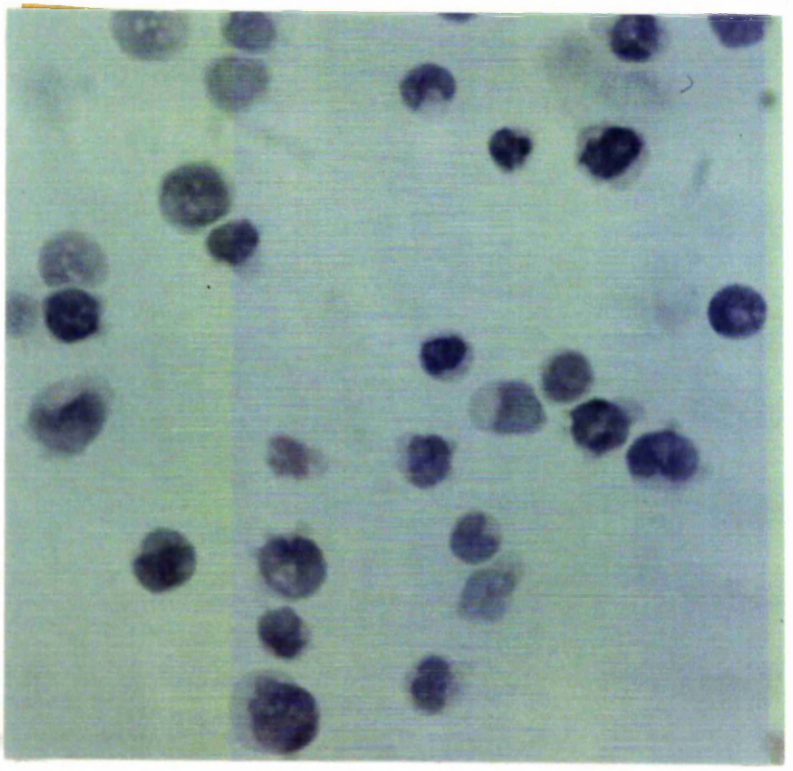
The percent $(EBNA)^+$ cells seemed somewhat low in this system. I think that this could have been due to the fact that the experiment was carried out in tissue culture tubes which have small surface areas. Overcrowding of the cells occurred, to limit the percentage viability of the culture. However, tissue culture tubes were most suitable for this

Fig. 1 Kinetics of the transformed state



experiment as it was being conducted over a long period of time (30 days), thus limiting the probability of contamination of the cultures.

Fig. 1D. B9 transformed lymphoblastoid cells from HUCB : stained by Giemsa's staining technique (Methods, 2.3); photographed with Ektachrome 50. (Magnification x 40).



1.3. Transforming Efficiencies of EBV B958 and EBV QIMR-WIL.

1.3.1. Introduction

Suspensions of EBV B958 and EBV QIMR-WIL were diluted ten-fold in freshly prepared CM (10^{-1} - 10^{-5}). Mononuclear cells were collected from HUCB samples by Ficoll-hypaque density gradient centrifugation and the cell densities brought to 2.4 x 10^6 viable cells/ml with freshly prepared CM. Equal volumes of cell suspensions from each of 6 HUCB samples were mixed with each dilution of virus in turn. Equal volumes of cell suspensions and CM (without added virus) were also mixed. The suspensions were incubated at 37°C, shaking the flasks every 30 mins. After 90 mins, the cells were collected by centrifugation at 200 xg for 10 mins. Aliquots (1.0 ml) were placed in tissue culture tubes (x4 for each virus dilution), and incubated at 37°C. The tubes were observed daily for evidence of the transformed state (Methods, 6.2). Half of the culture medium in each tube was replaced by freshly prepared CM every 3 days for 6 weeks or until the transformed state was evident. The transformed state was never evident in uninfected cultures.

1.3.2. Calculation of Transforming Efficiencies.

The transforming efficiency (TE) for each virus strain was used to describe the reciprocal of the virus dilution which induced 50% of replicate cultures to proliferate.

Here, 50% of replicate cultures was represented by 2 tubes. TE was calculated from the data in Table 1.2, by the following formula (Reed et al., 1938) :-

$$TE = \frac{\text{No. of transformed cultures above 50\%} - 50}{\text{No. of transformed cultures above 50\%} - \text{No. of transformed cultures below 50\%}}$$

Table 2 displays the data which was collated from the 12 HUCB samples when infected by EBV B958 and EBV QIMR-WIL. TE for EBV B958 and EBV QIMR-WIL were determined to be $10^{3.4}$ and $10^{1.0}$ TD_{50}/ml respectively.

Table 1.2. Transforming Efficiency Determination of EBV B958 and EBV QIMR-WIL.

EBV Strain	RLD	NIC	NUC	CIN	CUN	FIN	%IN
B958	1	24	0	70	0	$^{70}/_{70}$	100
	2	22	2	46	2	$^{46}/_{48}$	95.8
	3	18	6	24	8	$^{24}/_{32}$	75.0
	4	6	18	6	26	$^{6}/_{32}$	18.8
	5	0	24	0	50	$^{0}/_{50}$	0.0
QIMR-WIL	1	8	16	16	16	$^{16}/_{32}$	50
	2	5	19	8	35	$^{8}/_{43}$	18.6
	3	3	21	3	56	$^{3}/_{59}$	5.1
	4	0	24	0	80	$^{0}/_{80}$	0.0
	5	0	24	0	104	$^{0}/_{104}$	0.0

Abbreviations :-

RLD = Reciprocal log dilution

NIC = Number of infected cultures

NUC = " " uninfected cultures

CIN = Cumulative infected

CUN = Cumulative uninfected

FIN = Fraction infected

%IN = Percent infected

1.4. Electron Microscopy Studies on Producer Cells

1.4.1. Introduction

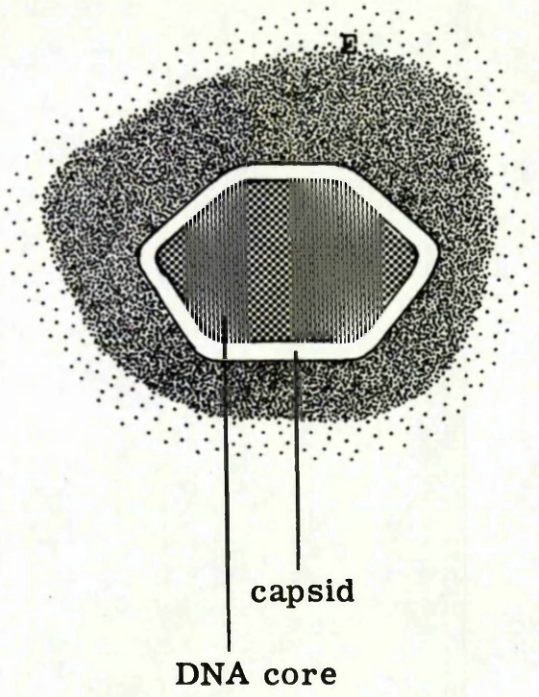
The viabilities of cultures of B958 and QIMR-WIL producer cell lines were determined by the Trypan blue exclusion test. Cells from cultures which were > 90% viable were suspended in freshly prepared CM (3×10^5 cells/ml). After 9 days incubation at $37^{\circ}C$, cell pellets were collected by centrifugation at 350 xg for 20 mins. The pellets were fixed, dehydrated, sectioned, and observed by electron microscopy (EM) as detailed in Section 4 (Methods).

A 200 mesh with 100 squares was used on EM grids. Approximately 25 squares, (20 cells/square) were investigated from 10 samples of pellets of B958 or QIMR-WIL cultures. Therefore, for each producer cell line, approx. 5,000 cells were investigated.

1.4.2. Discussion

Figs. 2-7 illustrate and summarize typical electron micrographs which were observed. In Fig. 2, E = viral envelope.

Fig. 2 Structure of Encapsidated EBV Particle



(Magnification x 81,000)

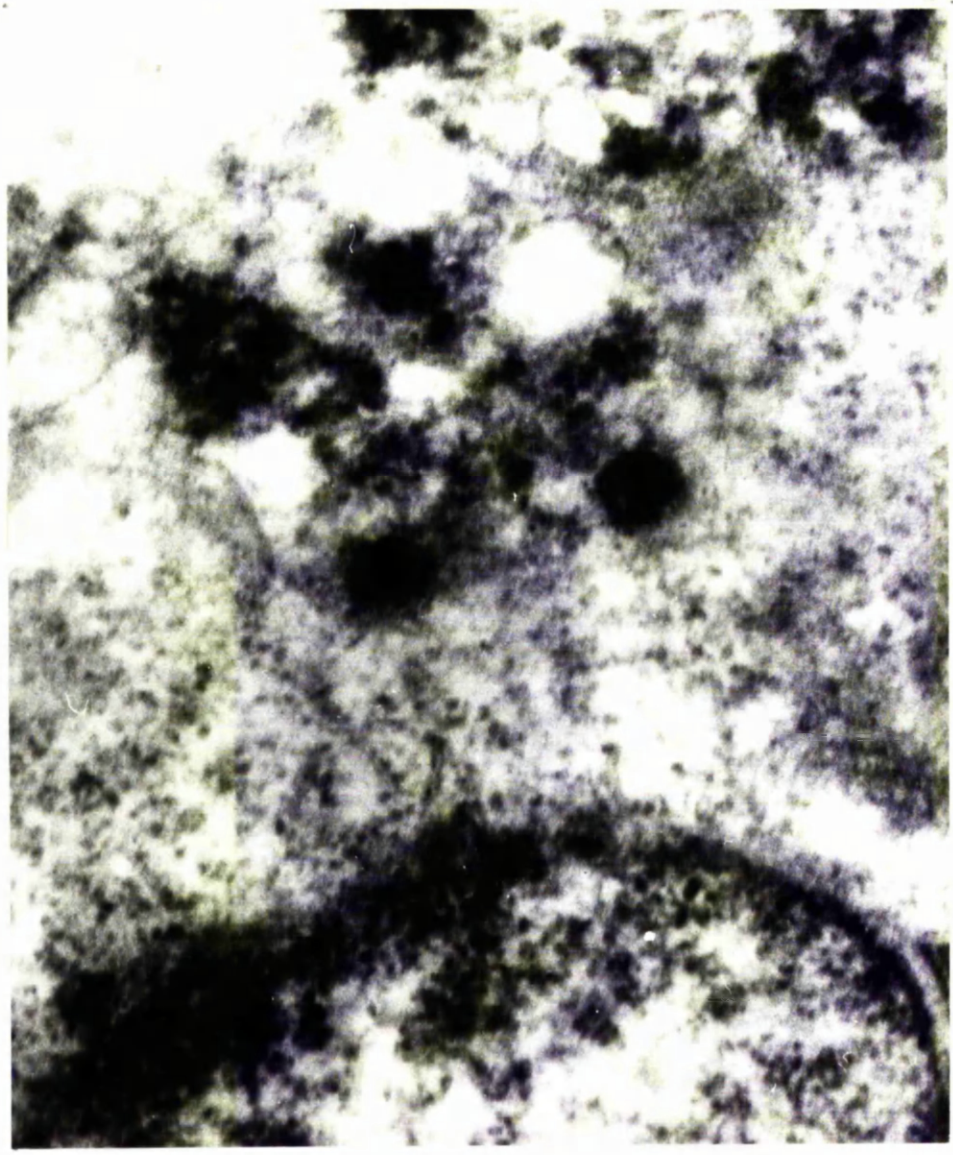


Fig. 3. Partially disrupted B958 Cell

Encapsidated forms of EBV B958 can be seen in the cytoplasmic matrix of the partially degenerated cell.

T = tubular structure (Kitamura et al., (1978))

E = Early stage in the replication of Herpesviruses, showing viral capsid with bar (Friedmann et al., (1975))

(Magnification x 39,000).

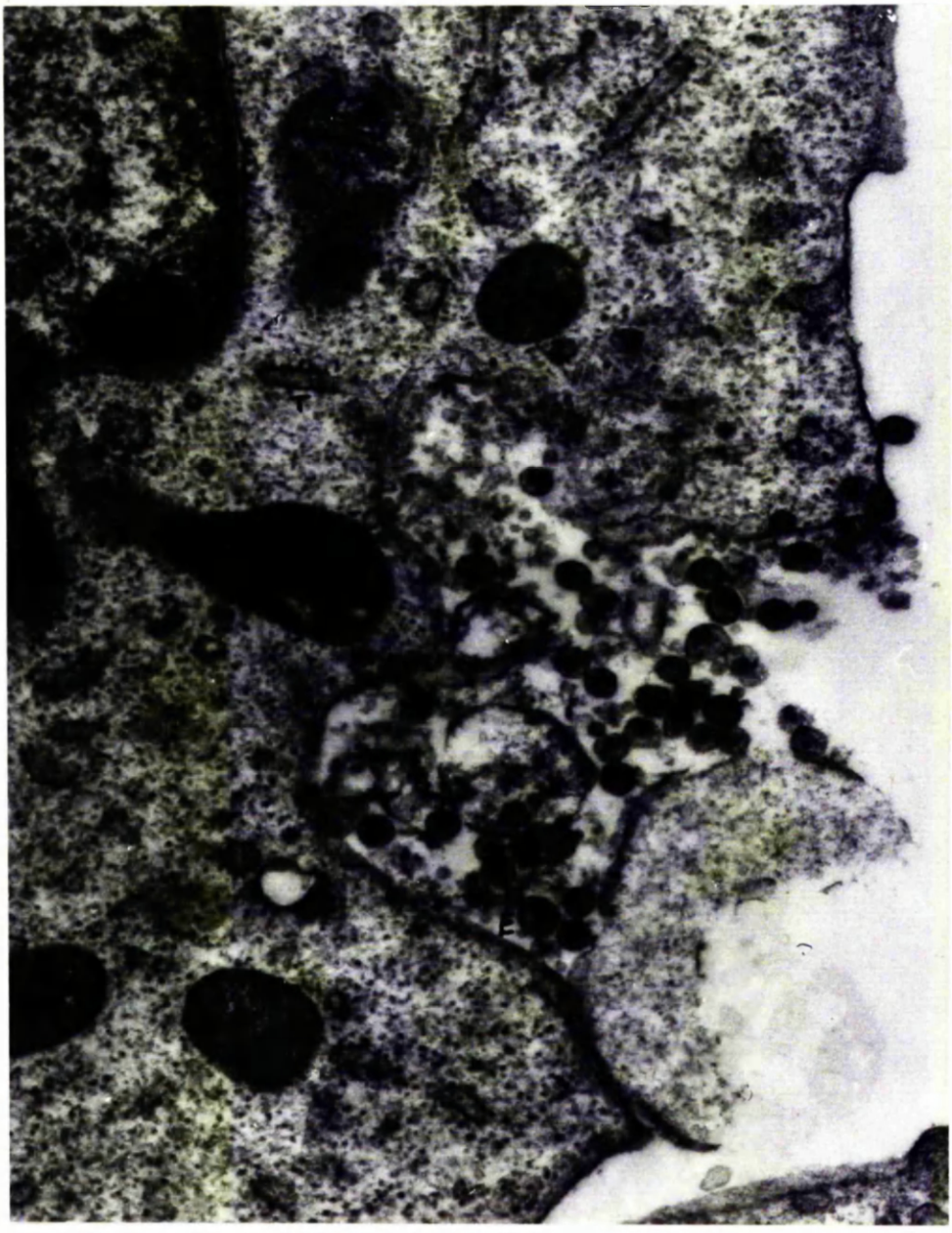


Fig. 4. Degenerated form of QIMR-WIL Cell

Many empty capsids can be seen in the nuclear and cytoplasmic matrices of the cell. Arrows in Fig. represent extracellular forms of EBV QIMR-WIL. The encapsidated DNA cores are early stages in the replication of Herpesviruses. (Magnification x 20,050).

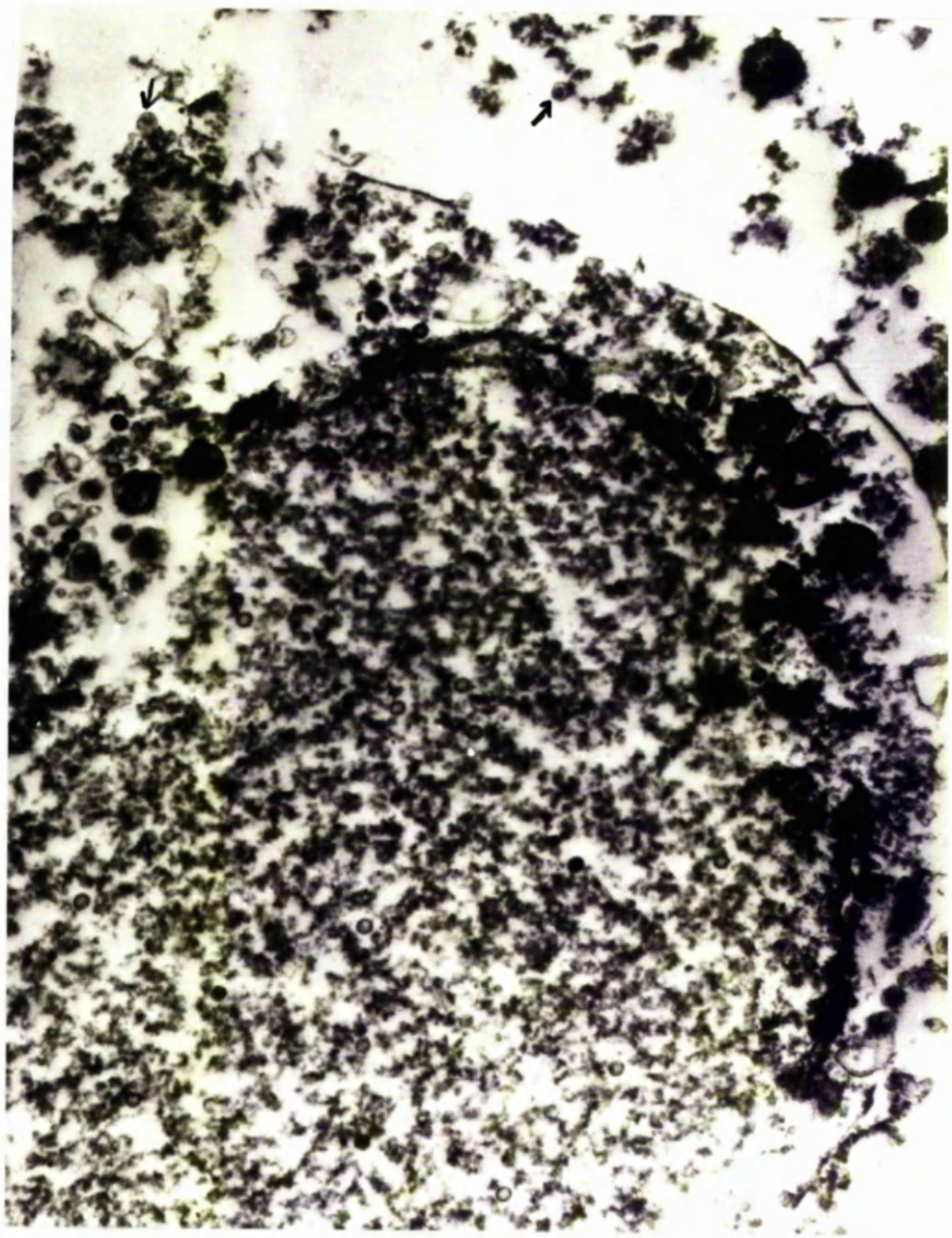


Fig. 5. Partially Disrupted form of QIMR-WIL Cell

Arrows indicate various forms of encapsidated EBV QIMR-WIL. Two forms of EBV QIMR-WIL (large arrows in the cytoplasmic matrix) are seen with the viral DNA filaments not completely within the viral capsids. Ringed arrow indicates an encapsidated, barred, form of EBV QIMR-WIL. (Magnification x 39,000).

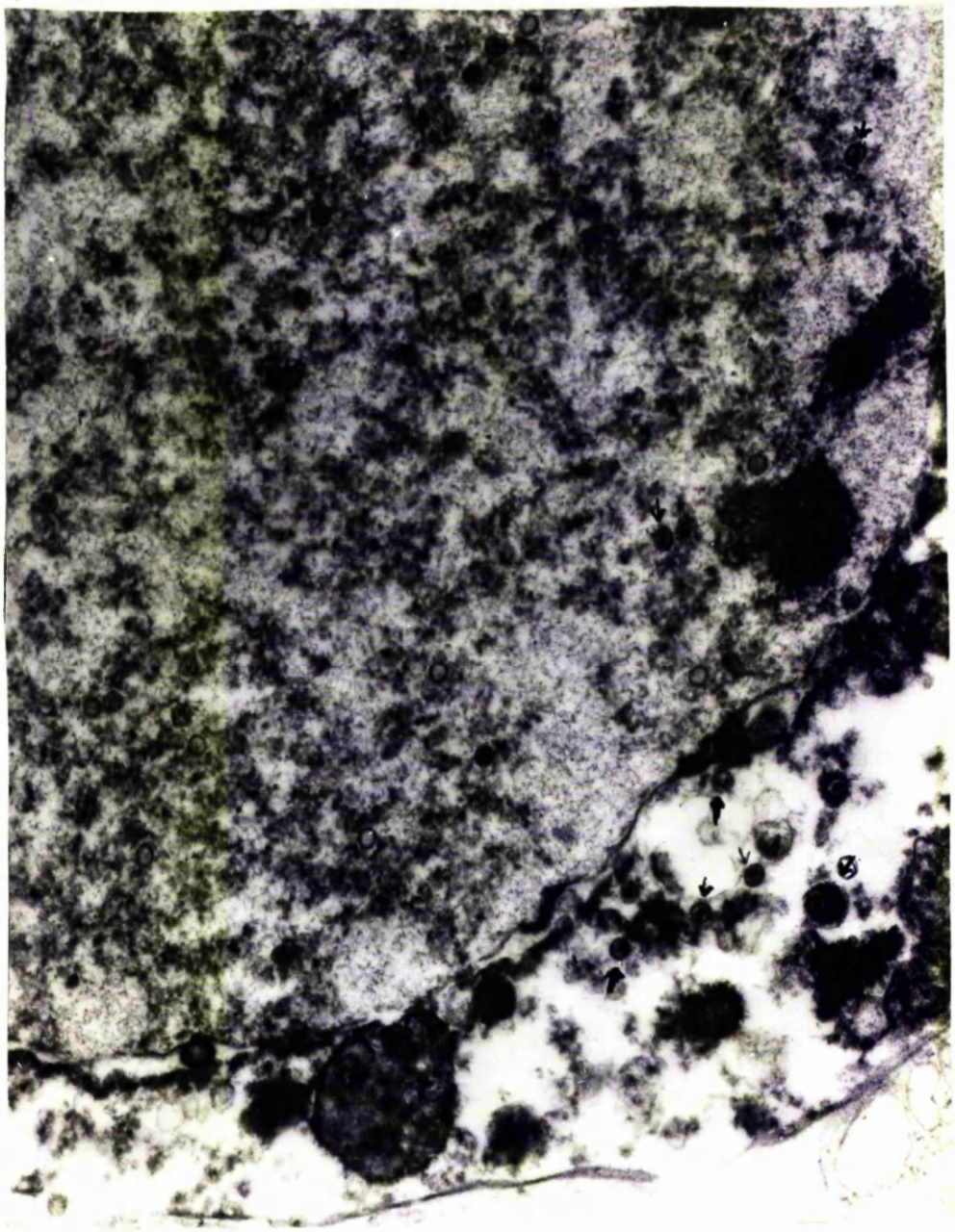
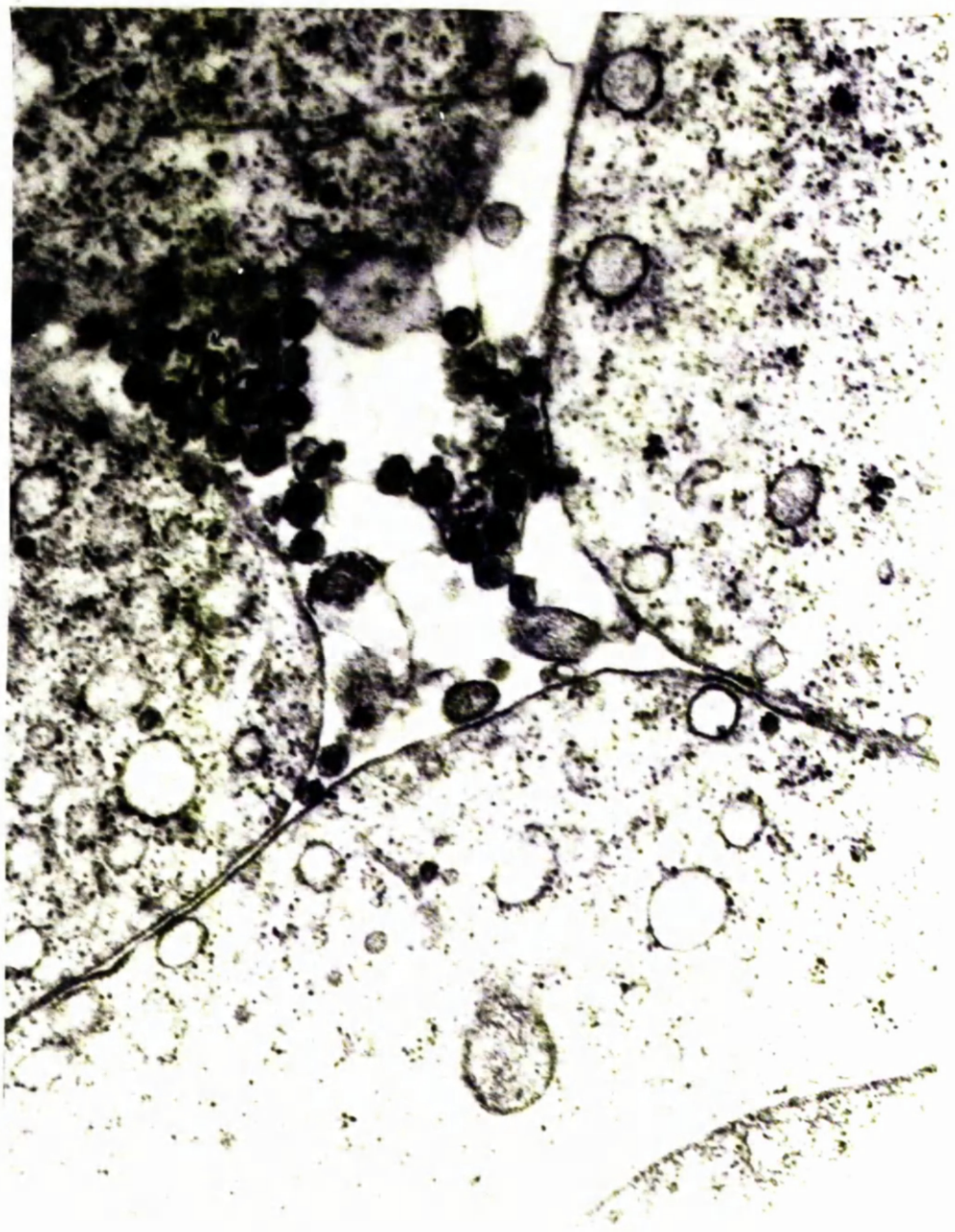


Fig. 6. Undisrupted form of B958 Cells

The plasma membranes of B958 cells have not been disrupted after 9 days of cultivation. Two encapsidated EBV B958 particles are seen attached to the cell on the right. Attachment of the viral particles to the cell could be preceeding penetration of the cell. (Magnification x 66,000).

Fig. 7. Extracellular forms of EBV B958

A viral DNA filament is seen partially encapsidated on the surface of a B958 cell (top, left). Many encapsidated forms of EBV B958 are evident. Two capsids are seen with 5 DNA cores encapsidated, suggesting >1 EBV (B958) DNA core can become encapsidated in the capsids of EBV B958 particles. (Magnification x 40,050).



1.5. Population Doubling Times for EBV-transformed Cells

1.5.1. Introduction

EBV B958 and EBV QIMR-WIL transformed cells were maintained in culture for 4 (-SAM78, -EIF78) or 6 (-IM77, -RK77) months after the establishment of the transformed state. The viabilities of the pairs of cell lines were determined by the Trypan blue exclusion test. Cultures of cells which were 90-93% viable, were suspended in CM (10.0 mls) at 3-3.5 x 10^5 cells/ml - although the densities varied, care was taken to ensure that each pair of cell line had been suspended at similar densities. The cell suspensions were duplicated for each cell line in tissue culture flasks (25 cm^2) and incubated at 37°C.

Samples (200 µl) were removed from each cell suspension at the following times after incubation :-

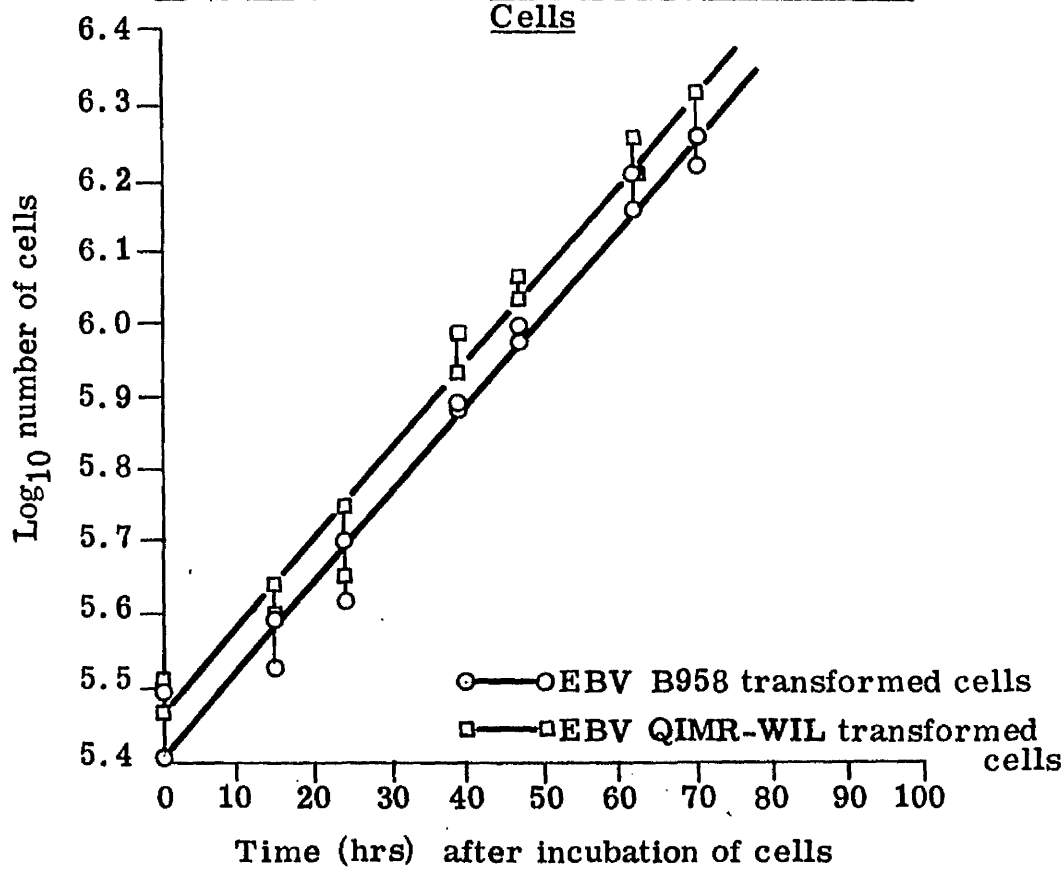
0, 15, 24, 39, 47, 62 and 70 hrs.

Clumps of cells were gently disrupted before each sample was taken and the viability of the sample determined by the Trypan blue exclusion test.

1.5.2. Calculation of Population Doubling Times : Discussion

The data in Fig. 8 represents $\log_{10}$ No. of cells at time t (hrs) after infection. Each point on the graph represents an average of 4 cell lines - the culture for

Fig. 8 Kinetics of growth of EBV-transformed Cells



each cell line was duplicately sampled. Regression lines were determined and the slopes of the graphs calculated according to the method of Bishop (1978). The slopes of the lines were determined to be 0.12 for B9 and QW transformed cell lines. This suggested that the growth rates were similar for both groups of cell lines.

The growth rate constant (k) for each cell line was determined mathematically, by making use of the following formula (Paul, 1975) :-

$$K = \frac{\text{Log}_{10}N_t - \text{Log}_{10}N_0}{0.301t}$$

where : $\frac{\text{Log}_{10}N_t - \text{Log}_{10}N_0}{t}$ = slope of line

$\text{Log}_{10}N_0$ = No. of cells at time 0 hrs.

$\text{Log}_{10}N_t$ = No. of cells at time t hrs.

t = 47 hrs; 0.301 = growth rate factor.

The population doubling time X = 1/k hrs.

The averages of X for QW transformed cell lines which had been maintained in culture for 4 and 6 months were 26.52 and 27.72 hrs respectively. The averages of X for B9 transformed cell lines which had been maintained in culture for 4 and 6 months were 29.39 and 28.60 hrs respectively :-

B9 transformed		QW transformed	
Cell Line	x (hrs)	Cell Line	x (hrs)
*B9SAM78	34.48	*QWSAM78	22.73
*B9EIF78	24.29	*QWEIF78	30.30
B9IM77	27.29	QWIM77	24.39
B9RK77	29.41	QWRK77	31.25

* Maintained in culture for 4 months.

Raji (6HAT25DC), B958 and QIMR-WIL were subjected to the same experimental conditions as described in the Introduction of this section. The populations' doubling times were determined for each of the cell lines. X was found to be consistently 22.7, 21.4, and 32.6 hrs for Raji, B958, and QIMR-WIL cell lines respectively. X for the 4 B9 and QW transformed cell lines were determined to be 28.87 and 27.18 hrs respectively.

There was no statistically significant difference observed between X for cultures of B9 or QW transformed cells which had been maintained in culture for 4 or 6 months after establishment of the transformed state, or between pairs of B9 and QW transformed cell lines. X was therefore a stable factor among producers (B958, QIMR-WIL), restringently infected (Raji) and EBV-transformed HUCLs.

1.6. Population Densities of EBV-transformed Cell Lines

1.6.1. Introduction

B9 and QW transformed cell lines were maintained in culture for 7 or 5 months after the establishment of the transformed state. The viabilities of cultures were determined by the Trypan blue exclusion test. Cell cultures which were >90% viable were suspended in CM (3-3.5 x 10^5 cells/ml); pairs of cell lines were suspended at similar densities. Samples (200 μl) were removed from duplicate culture flasks (for each cell line under test) over a period of 10 days at the following time intervals :-

0, 1, 2, 3, 4, 5, 7 and 10 days.

The viability of each sample was determined - before sampling, clumps of cells in suspension were disrupted. The population densities (Pds), were determined at the time of sampling each cell line. Table 1.3 displays the maximum population densities (P_{max}) which were attained and the time (t, in days (d)) at which P_{max} was attained for each cell line.

1.6.2. Discussion

Among B9 transformed cell lines, the averages of P_{max} for 5 and 7 month-old cultures were 14.66 and 13.36 x 10^5 cells/ml respectively. Among QW transformed cell lines, the averages of P_{max} for 5 and 7 month-old cultures were 19.75 and 20.34 x 10^5 cells/ml respectively. Older

cultures of B9 transformed cell lines therefore had fractionally lower, while older cultures of QW transformed cell lines had fractionally higher densities than newly established cultures.

Among B9 and QW transformed cell lines, P_{max} for 11 pairs of cell lines were 14.07 ± 0.94 and $19.01 \pm 1.16 \times 10$ cells/ml respectively. P_{max} were attained on :-

2(1), 3(7), 4(3) and 3(4), 4(7) days

respectively for B9 and QW transformed cell lines - where the numbers in brackets represent the number of cell lines. QW transformed cell lines had longer life span in culture than B9 transformed cell lines, thus acquiring a capacity to attain higher population densities.

Raji (6HAT25DC), QIMR-WIL, and B958 cell lines were suspended in freshly prepared CM (3×10^5 cells/ml) and subjected to similar experimental conditions as detailed in 1.6.1. of this section. The growth patterns of the producer cell lines were represented graphically (Fig. 9). Averages of the Pds which were attained at each time interval for the 11 pairs of cell lines which had been investigated, were also represented graphically on Fig. 9 : Pd (number of cells $\times 10^5$/ml) against t (days).

B958 cells survived for a much longer period of time in culture than QIMR-WIL cells. QIMR-WIL cells on the other hand, reached higher P_{max} (30×10^5 cells/ml) than B958 cells (26.2×10^5/ml). Raji (6HAT25DC) consistently

Table 1.3. Population Densities of EBV-transformed Cell Lines

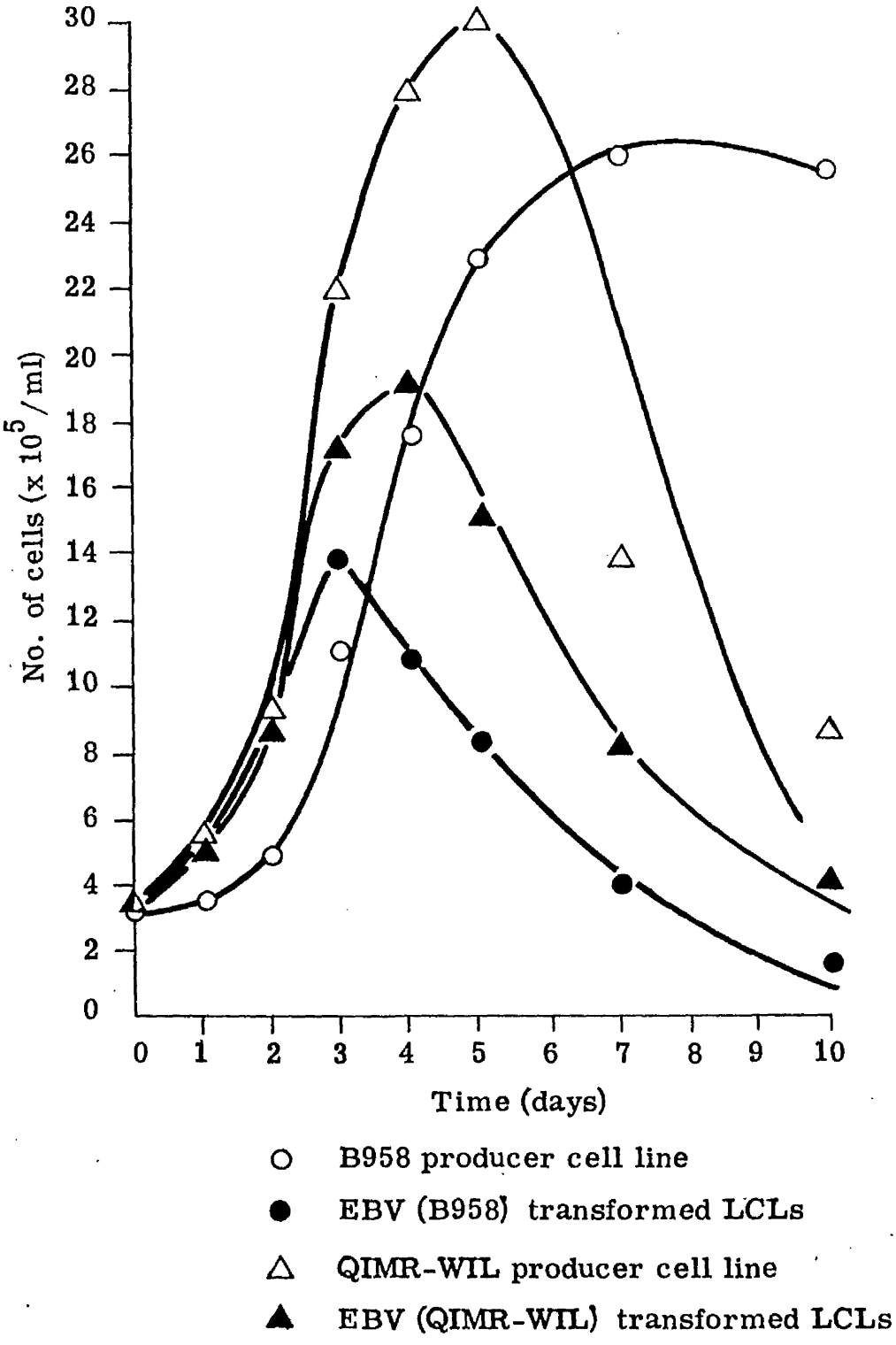
B9 Transformed Cell Line	P_{max} ($\times 10^5$/ml)	T(d)	QW Transformed Cell Line	P_{max} ($\times 10^5$/ml)	T(d)
*B9SAM78	16.6	3	*QWSAM78	20.2	4
*B9ELB78	13.1	2	*QWELB78	14.4	3
*B9EIF78	15.8	3	*QWEIF78	19.3	4
*B9MAC78	13.6	3	*QWMAC78	22.8	4
*B9AMR78	8.7	3	*QWAMR78	18.3	4
*B9LOC78	20.2	4	*QWLOC78	23.5	4
B9FL77	13.5	3	QWFL77	20.9	3
B9AW77	11.0	4	QWAW77	22.6	3
B9RK77	15.3	3	QWRK77	23.7	4
B9AB77	13.0	4	QWAB77	14.4	4
B9SD77	14.0	3	QWSD77	20.1	3

*Cell lines maintained in culture for 5 months after establishment of the transformed state - other cell lines were maintained for 7 months.

had P_{max} at 25.8 x 10^5 cells/ml, suggesting that P_{max} once acquired was a stable feature of the cell line, under consistent experimental conditions. Holly (1975) suggested that P_{max} *in vitro* could be a measure of tumourgenicity *in vivo*. P_{max} was consistently attained on the fourth day after incubation for Raji and QW transformed cells. QW transformed cell lines could therefore represent populations of EBV-transformed cells which are nearer to the tumourigenic state than B9 transformed cells, in terms of proliferative capacity.

The fact that B958 survives for a much longer period of time in culture would suggest that an advantageous characteristic is conferred on monolayer cultures like B958. The fact that most EBV-transformed LCLs in culture proliferate as clumps of cells, would suggest that cell clumping could be an alternative to the adherence phenomenon of monolayer cultures, for survival *in vitro*. Degeneration of the culture could be associated with loss of adherence or cell clumping capacities.

Fig. 9 Growth patterns of EBV-transformed Cells



1.7. Summary

Encapsidation of Herpesviruses occurs within the nuclei of infected cells. Assembly of the viral DNA occurs in the nuclei and passes from the nucleoplasm into the preformed capsid, via pervious pores in the capsids (Friedmann et al., (1975)). Envelopment of the encapsidated particle occurs as the viral particle passes from the nucleus to the cytoplasm for subsequent release. Envelopment is a feature of Herpesviruses but is not a prerequisite for the release of viral particles.

Many EBV B958 particles were seen in the extracellular matrices with complete hexagonal EBV (B958) DNA cores. On the other hand, most EBV (QIMR-WIL) DNA cores which were observed in electron micrographs were incomplete. In Fig. 5 (large arrows) partially encapsidated forms of EBV QIMR-WIL were evident. In Fig. 2., an enveloped, cytoplasmic form of EBV QIMR-WIL was evident, which would suggest that EBV QIMR-WIL do become enveloped on occasions. Envelopment of encapsidated EBV QIMR-WIL is probably a prerequisite for the release of fully encapsidated, hexagonal, EBV (QIMR-WIL) DNA cores. On the other hand, envelopment of encapsidated EBV B958 is probably not a prerequisite for the release of fully encapsidated forms of EBV (B958) DNA cores.

Henderson et al., (1977) found that marmoset lymphocytes were 1,000 times less sensitive to EBV (B958) transformation than HUCLs. Yet, Miller et al., (1973) obtained the high

transforming EBV B958 strain by passaging EBV 883-L in B958 cells. The fact that more than one EBV (DNA) core can become encapsidated in EBV particles could contribute to the high transforming efficiency of EBV B958. The next stage in these studies would be to analyze the extracellular forms of EBV 883-L, for comparison with the extracellular forms of EBV B958. EBV B958 could be a mutant strain of EBV 883-L which reverts to the wild type with lower transforming potential in marmoset cells.

The transforming efficiencies of EBV B958 and EBV QIMR-WIL were found to be $10^{3.4}$ and $10^{1.0}$ TD_{50}/ml respectively. The time lapse between infection and establishment of the transformed state for pairs of B9 and QW infected cells were found to be 12.8 ± 0.5 and 24.2 ± 1.5 days respectively. The lower TE for EBV QIMR-WIL, therefore influenced the longer time lapse which was observed between infection and establishment of the transformed state among QW infected cells.

Chapter 2

Immunological Features of EBV-transformed Cells

2.1. EBV-associated Antigens In Pairs of Transformed Cell Lines.

2.1.1. Introduction

The 16 pairs of B9 and QW transformed cell lines were maintained in cultures for 3-5 months after the establishment of the transformed state had been determined. The viabilities of cultures from 2 day-old cultures were determined by the Trypan blue exclusion test (Methods, 2.2). Smears were prepared from cultures which were >90% viable by cytocentrifugation (Methods, 2.2).

EBNA was detected by the anticomplementary immunofluorescence test on acetone/methanol-fixed smears (Methods, 9.4). Smears of cells from cultures and suspensions of Raji, BJAB and uninfected HUCL preparations were prepared and tested as positive and negative controls for EBNA. $(EBNA)^+$ cells were regularly detected among Raji but never among BJAB or uninfected HUCL preparations. EA or VCA was detected by direct IF test on acetone-fixed smears (Methods, 9.3.3). Smears from Raji and P_3HR-1 cultures were used as negative and positive controls respectively in the direct IF test for the detection of EA and VCA positive cells. EA and VCA positive cells (2-3%) were always detected among P_3HR-1 but never among Raji cells.

The percentages of EBNA, EA and VCA positive cells in each of the 16 pairs of cell lines were determined (Methods, 9.1). Photographs of smears of cells which were treated for the detection of EBNA and EA, were taken with high speed Ektachrome 400 film (Kodak) with u.v.

illumination (Figs. 10-15). The patterns of fluorescence were similar between EA and VCA positive cells.

2.1.2. Synthesis of EBNA Among Pairs of Cell Lines

All 16 pairs of cell lines which had been B9 and QW transformed, synthesized EBNA (Table 2.1). 81.7 - 97.6% and 88.5 - 97.6% were $(EBNA)^+$ among QW and B9 transformed cells respectively. The average percent $(EBNA)^+$ cells among the 16 pairs of cell lines were 91.6% and 89.88% for B9 and QW transformed cell lines respectively.

The maximum population densities (P_{max}) among B9 transformed cell lines were attained on the third day after subculture. On the other hand, P_{max} for most QW transformed cell lines tested (Section 1.6, Results) was attained on the fourth day after subculture. EBNA is at its maximal level among EBV-transformed cells during late G_1/early S_1 phases of the cell cycle (Slovin et al., (1980); Ernberg et al., (1977)). EBNA detection was carried out among both B9 and QW transformed cells on the second day after subculture. The fractional differences which were observed between the $(EBNA)^+$ cells of B9 and QW transformed cell lines (Table 2.1), most probably reflected the different stages in the cell cycle, among the two groups of cell populations.

The pattern of fluorescence among $(EBNA)^+$ cells varied from finely granular to brightly staining. The pattern of fluorescence was similar between pairs of cell

Table 2.1 EBV-associated Antigen Positive Cell Lines

B9 Transformed Cell Lines				QW Transformed Cell Lines			
Cell Line	% Positive Cells for			Cell Line	% Positive Cells for		
	EBNA	EA	VCA		EBNA	EA	VCA
B9SD77	94.1	-	-	QWSD77	87.2	-	-
B9FL77	89.7	-	-	QWFL77	85.1	-	-
B9MM77	92.8	-	-	QWMM77	87.6	-	-
B9RK77	88.5	1.4	-	QWRK77	95.8	-	-
B9EL77	90.6	1.6	-	QWEL77	85.6	0.3	-
B9IM77	92.4	-	-	QWIM77	82.1	-	-
B9AW77	97.6	-	-	QWAW77	90.2	-	-
B9AB77	90.1	-	-	QWAB77	93.0	-	-
B9ELB78	89.1	-	-	QWELB78	88.1	-	-
B9EIF78	92.0	-	-	QWEIF78	84.8	-	-
B9SAM78	96.2	0.8	-	QWSAM78	81.7	-	-
B9BEB78	92.1	-	-	QWBEB78	94.7	-	-
B9LOC78	88.9	0.4	0.2	QWLOC78	95.7	-	-
B9MAC78	89.7	-	-	QWMAC78	96.1	-	-
B9EIB78	93.8	-	-	QWEIB78	97.6	-	-
B9AMR78	89.2	-	-	QWAMR78	92.8	-	-

Abbreviation : - = positive cells not detected.

lines which had been obtained from one pool of HUCLs (Figs. 10 and 11) as well as between pairs of cell lines which had been obtained from dissimilar pools of HUCLs (Figs. 12 and 13).

2.1.3. Producers Among Newly Established Cell Lines

EA was detected as a diffused component of $(EA)^+$ cells (Figs. 14 and 15). 4 cell lines (B9RK77, B9EL77, B9LOC78 and B9SAM78) which had been B9 transformed, synthesized EA. On the other hand, only one cell line (QWEL77) which had been QW transformed, synthesized EA. One pair of cell line, -EL77, synthesized EA. But, the percentage of EA synthesizing cells among B9EL77 population, was 5.3 times higher than the percentage EA synthesizing cells among QWEL77 population (Table 2.1, columns 3 and 7).

One cell line (B9LOC78) which had been B9 transformed, had 0.4% $(VCA)^+$ cells. On the other hand, neither QWLOC78 or any other QW transformed cell population, synthesized VCA. The percentage $(EA)^+$ cells among B9LOC78 population, was twice as many as the percentage $(VCA)^+$ cells.

B9 transformed cell lines therefore had higher numbers of producers among the 16 pairs of cell lines, than QW transformed cell lines. The kinetics of EA and VCA production was investigated.

2.1.4. Kinetics of EA and VCA Production

Cells from a culture of QIMR-WIL producer cell line which was >90% viable, were suspended in 50 ml freshly prepared CM (3×10^5 cells/ml) in sterile 100 ml conical flasks (x2). The cell suspensions were incubated at 37°C and samples (5.0 ml) were removed every 24 hrs on the following days after incubation :-

0, 1, 2, 3, 5, 8 and 10.

Smears were prepared from each sample by cytocentrifugation, treated for the detection of EA and VCA positive cells (Methods, 9.3.3) and the percentages EA and VCA positive cells determined from each smear (Methods, 9.1).

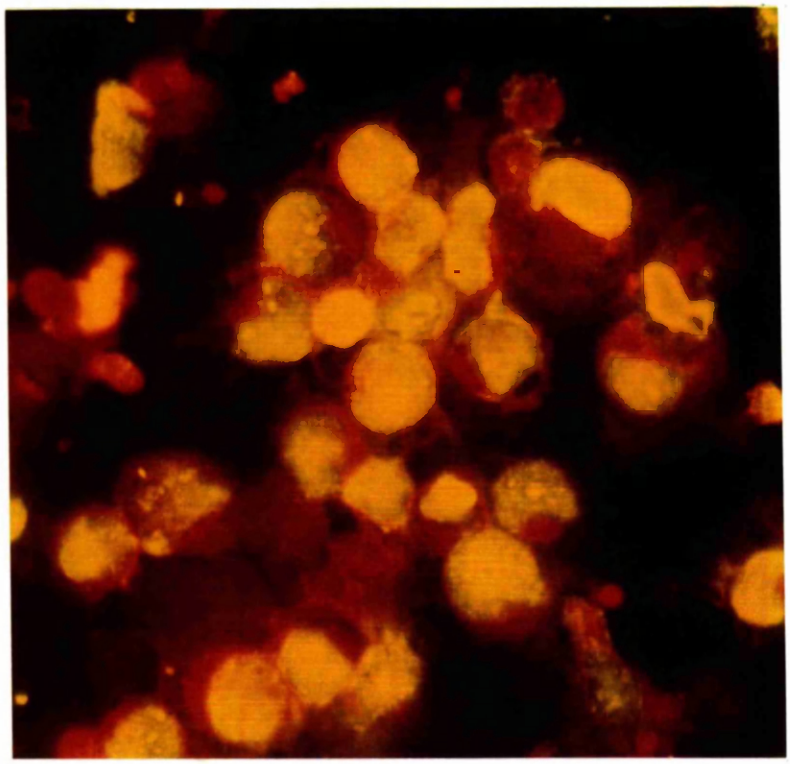
The percentages EA and VCA positive cells were found to increase with the time of incubation, and reached a maximal level on the second day after incubation of the cultures as indicated below :-

Time (days)	0	1	2	3	5	8	10
% EA^+ Cells	3.5	4.7	6.5	3.1	2.0	0.6	0.6
% VCA^+ Cells	1.9	2.2	2.5	1.8	0.0	0.0	0.0

Among producers, maximal EA and VCA syntheses were therefore achieved on the second day after subculture.

Fig. 10. <u>$(EBNA)^+$ cells among QW-transformed cells, QWSD77</u> : prepared by the anticomplementary immunogluorescence test (Methods, 9.4) and photographed with Ektachrome 400. (Magnification x 40).

Fig. 11. <u>$(EBNA)^+$ cells among B9-transformed cells, B9SD77</u> : prepared by the anticomplementary immunofluoresence test (Methods, 9.4) and photographed with Ektachrome 400. (Magnification x 40).



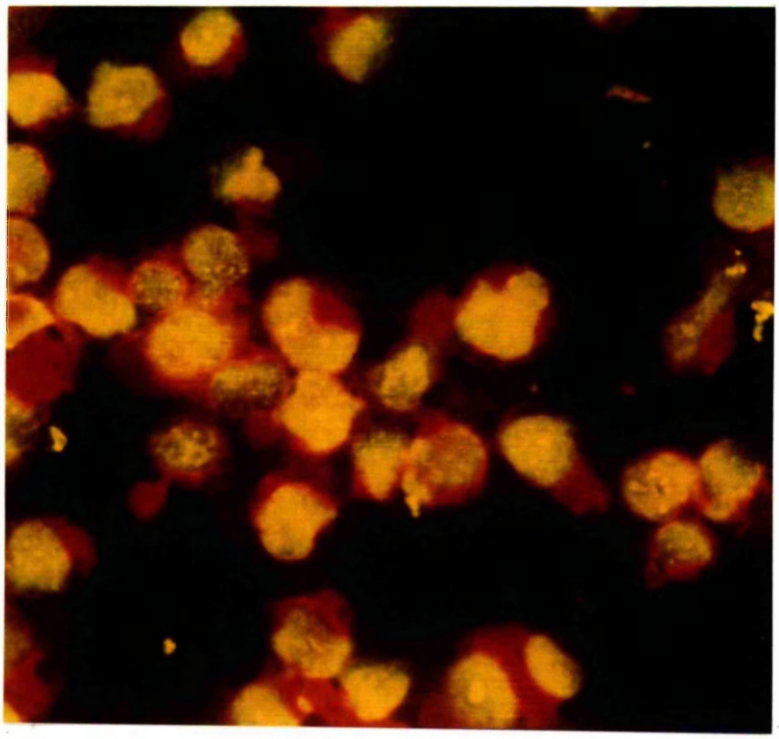
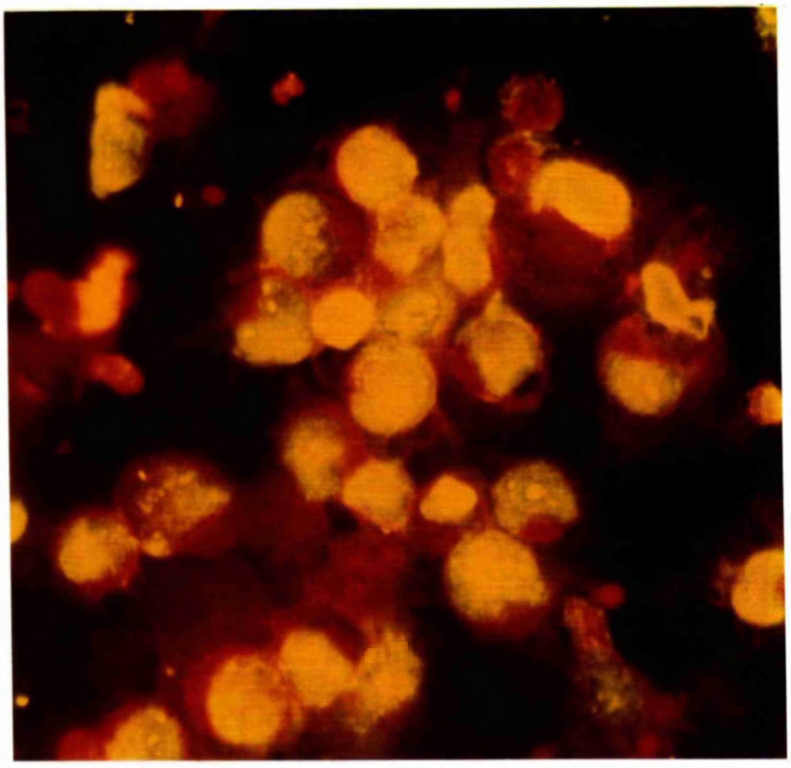
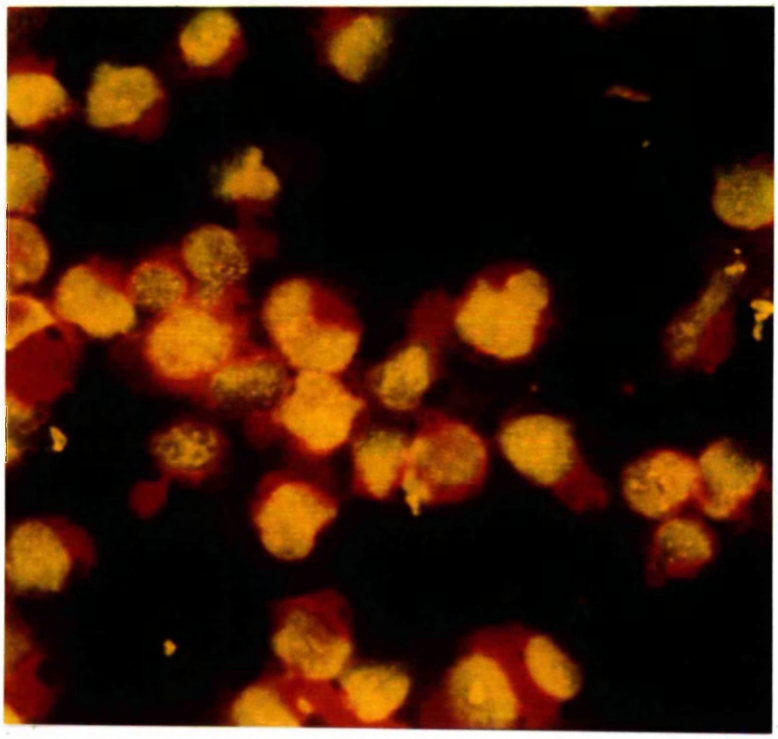
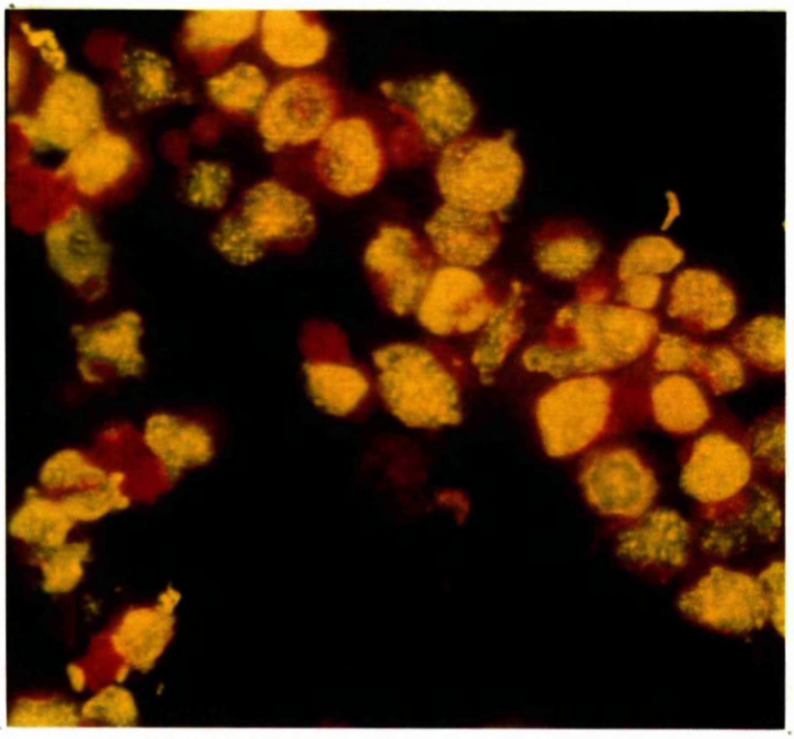


Fig. 12. $\underline{(EBNA)^+}$ $\underline{cells\ among\ QW\text{-}transformed\ cells,\ QWMM77}$: prepared by the anticomplementary immunofluorescence test (Methods, 9.4) and photographed with Ektachrome 400. (Magnification x 40).

Fig. 13. $\underline{(EBNA)^+}$ $\underline{cells\ among\ B9\text{-}transformed\ cells,\ B9ELB78}$: prepared by the anticomplementary immunofluorescence test (Methods, 9.4) and photographed with Ektachrome 400. (Magnification x 40).







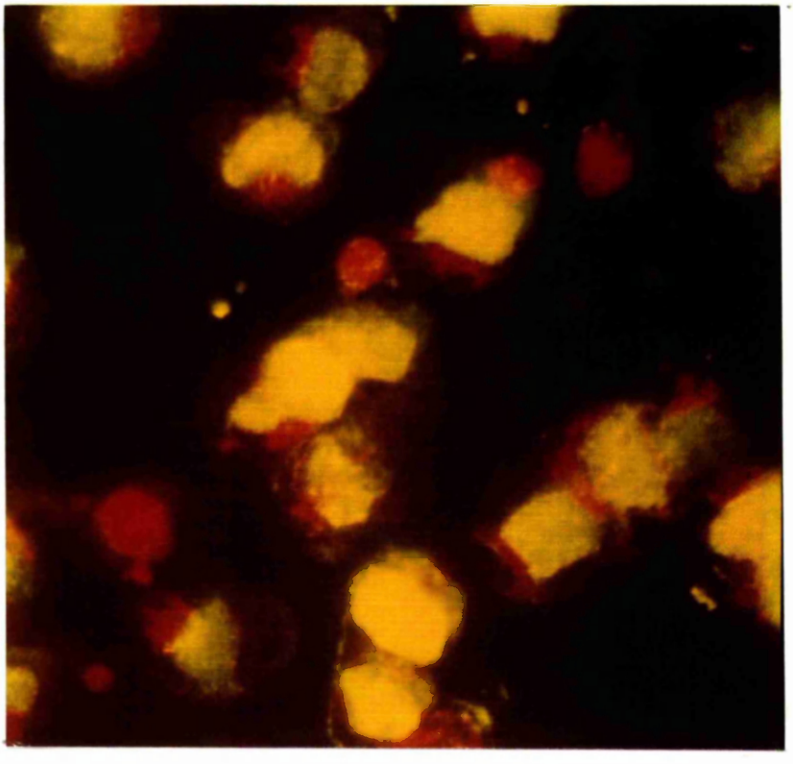
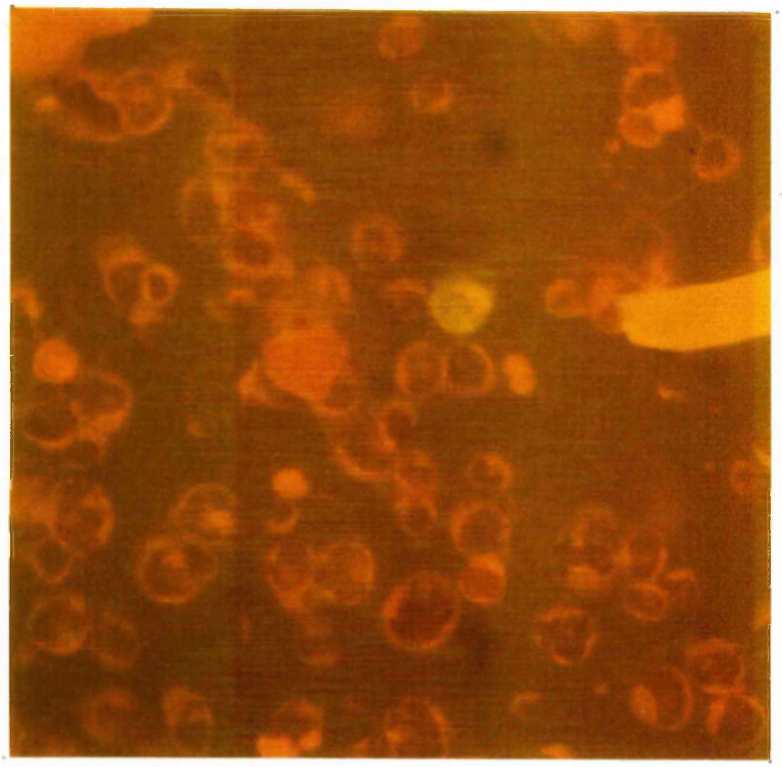
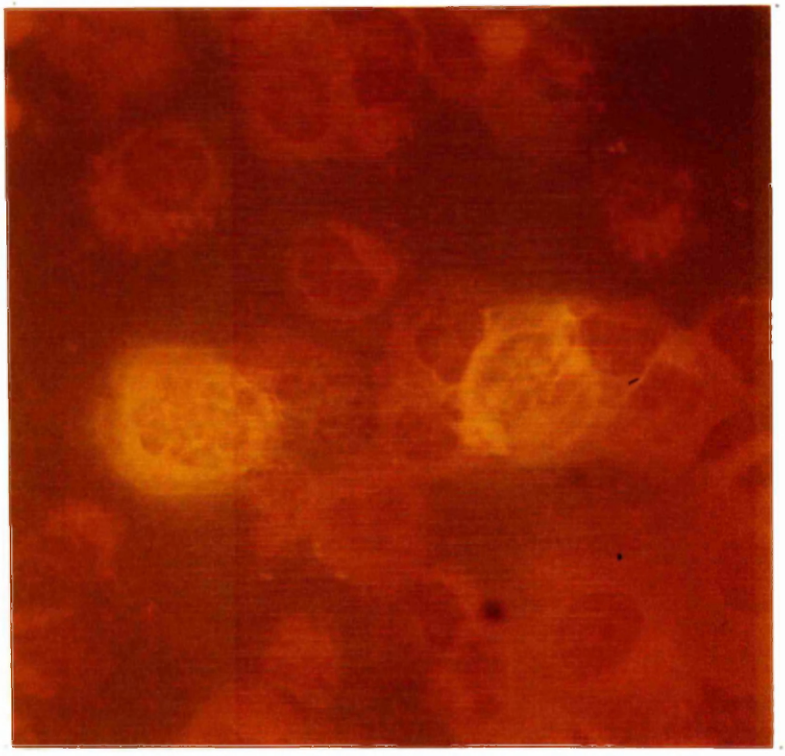


Fig. 14. $\underline{(EA)^+}$ cells among QW-transformed cells, QWEL77 : prepared by direct IF (Methods, 9.3.3) and photographed with Ektachrome 400. (Magnification x 25).

Fig. 15. $\underline{(EA)^+}$ cells among B9-transformed cells, B9RK77 : prepared by direct IF (Methods, 9.3.3) and photographed with Ektachrome 400. (Magnification x 100)





2.2. Ig Positive Cells Among Newly Established Cell Lines.

2.2.1. Introduction

The 16 pairs of cell lines were maintained in culture for 3-5 months after the transformed state had been established. Cell suspensions (>90% viable) from 2 and 3 day-old cultures were subjected to the direct IF test for the detection of cIg^+ (Methods, 9.3.2) and sIg^+ (Methods, 9.3.1) cells respectively.

sIg^+ cells were detectable mainly as discontinuous fluorescent rings (Fig. 16). The immunofluorescence of cIg^+ cells varied from detectable to bright (Fig. 17). The percentages of sIg^+ and cIg^+ cells were determined for the 16 pairs of cell lines.

2.2.2. Results and Discussion : Ig^+ Cells Within Cell Lines.

The averages of the percentages Ig^+ cells were determined as indicated :-

	Ig Type For							
	B9 transformed				QW transformed			
	μ	γ	k	λ	μ	γ	k	λ
sIg^+	19.2	6.3	13.2	3.6	11.5	3.5	7.7	3.5
cIg^+	12.1	2.6	4.7	4.4	3.7	1.3	1.4	1.5

The averages for the percent $sIg(\mu)^+$ and $sIg(\gamma)^+$ cells among B9 transformed cells were 19.2 and 6.3, but 11.5 and

3.5 among QW transformed cells. The ratios of the percentages $sIg(\mu)^+$: $sIg(\gamma)^+$ cells among B9 and QW transformed cells were 3.0 : 1.0 and 3.3 : 1.0 respectively. The percentages of $sIg(\mu)^+$ and $sIg(\gamma)^+$ cells among B9 transformed cells therefore exceeded those among QW transformed cells. However, the ratios of the percentages of $sIg(\mu)^+$: $sIg(\gamma)^+$ cells among B9 and QW transformed cells, approximated equality.

The percentage $sIg(k)^+$ cells among B9 transformed cells exceeded those among QW transformed cells. On the other hand, the percentages $sIg(\lambda)^+$ cells among both groups of transformed cells were similar. Among B9 and QW transformed cells, the percentages $sIg(k)^+$ cells exceeded the percentages $sIg(\lambda)^+$ cells. The ratios of $sIg(k)^+$: $sIg(\lambda)^+$ cells were found to be 3.7 : 1.0 and 2.2 : 1.0 among B9 and QW transformed cells respectively.

The ratios of $cIg(\mu)^+$: $cIg(\gamma)^+$ cells were; 4.7 : 1.0 and 2.7 : 1.0, among B9 and QW transformed cells respectively. The ratios of B9 : QW transformed cells for $cIg(\mu)^+$: $cIg(\gamma)^+$ cells were; 3.3 : 1.0 and 2.0 : 1.0 respectively. The percentages $cIg(\mu)^+$ cells therefore exceeded the percentages $cIg(\gamma)^+$ cells among B9 and QW transformed cells. The percentages $cIg(\mu)^+$ and $cIg(\gamma)^+$ cells among B9 transformed cells, exceeded those among QW transformed cells.

The percentages of $cIg(k)^+$ and $cIg(\lambda)^+$ cells among B9 transformed cells, exceeded those among QW transformed cells. The ratio of the percentages $cIg(k)^+$: $cIg(\lambda)^+$ cells was 1.1 : 1.0 among B9 transformed cells. Among QW transformed

cells, there was a slight imbalance in the ratio of $cIg(k)^+$: $cIg(\lambda)^+$ cells (0.9 : 1.0) in favour of $cIg(\lambda)^+$ cells.

The numbers of Ig^+ (both sIg^+ and cIg^+) cells among B9 transformed cells therefore exceeded the numbers of Ig^+ cells among QW transformed cells.

Among both B9 and QW transformed cell lines, the percentages and distributions of sIg^+ cells exceeded those for cIg^+ cells among Igs μ, γ, k and λ positive cells.

Fig. 16. <u>sIg$^+$ cell among QW-transformed cells, QWAW77</u> : prepared by direct IF (Methods, 9.3.1) and photographed with Ektachrome 400. (Magnification x40).

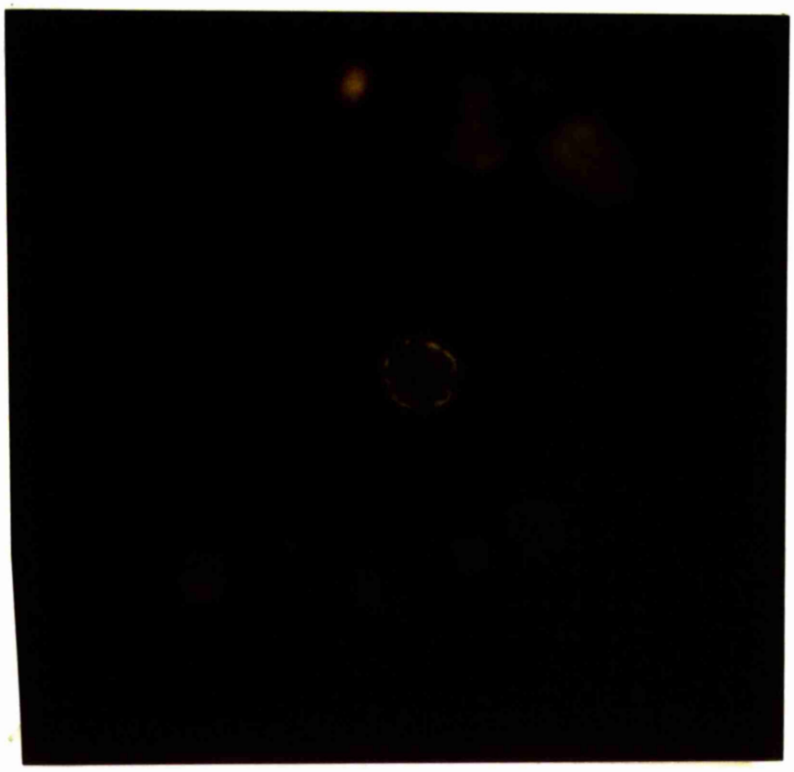
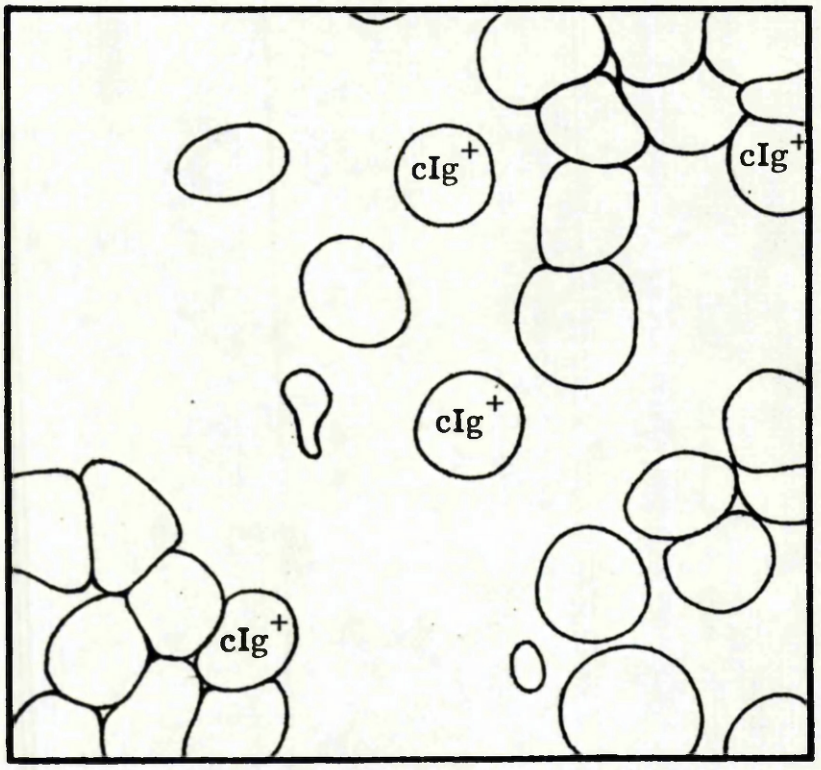
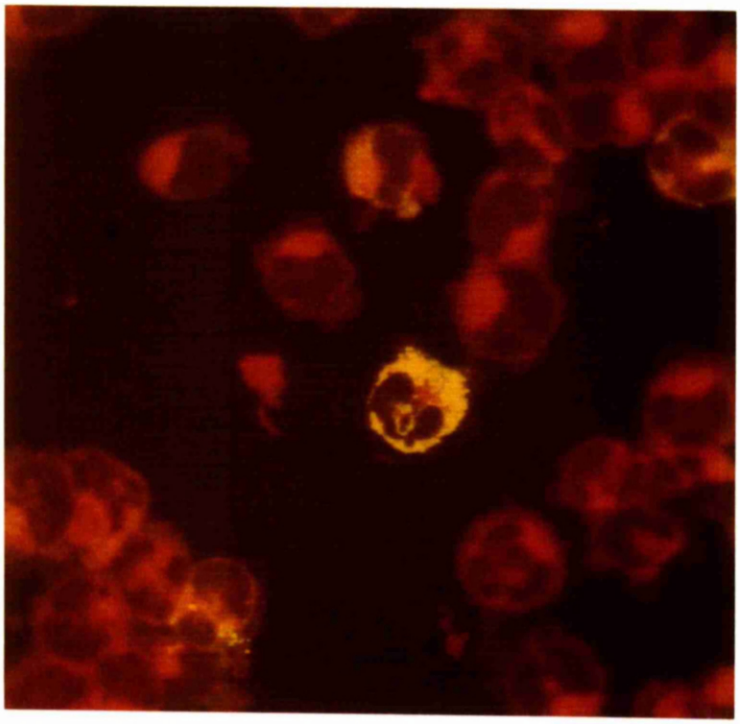


Fig. 17 Fluorescence of Immunoglobulin molecules within the cytoplasms of EBV-transformed cell line QWEIB78; detected by FITC-RAH Ig (μ) Ig type in the direct IF Technique

(Magnification x 40)





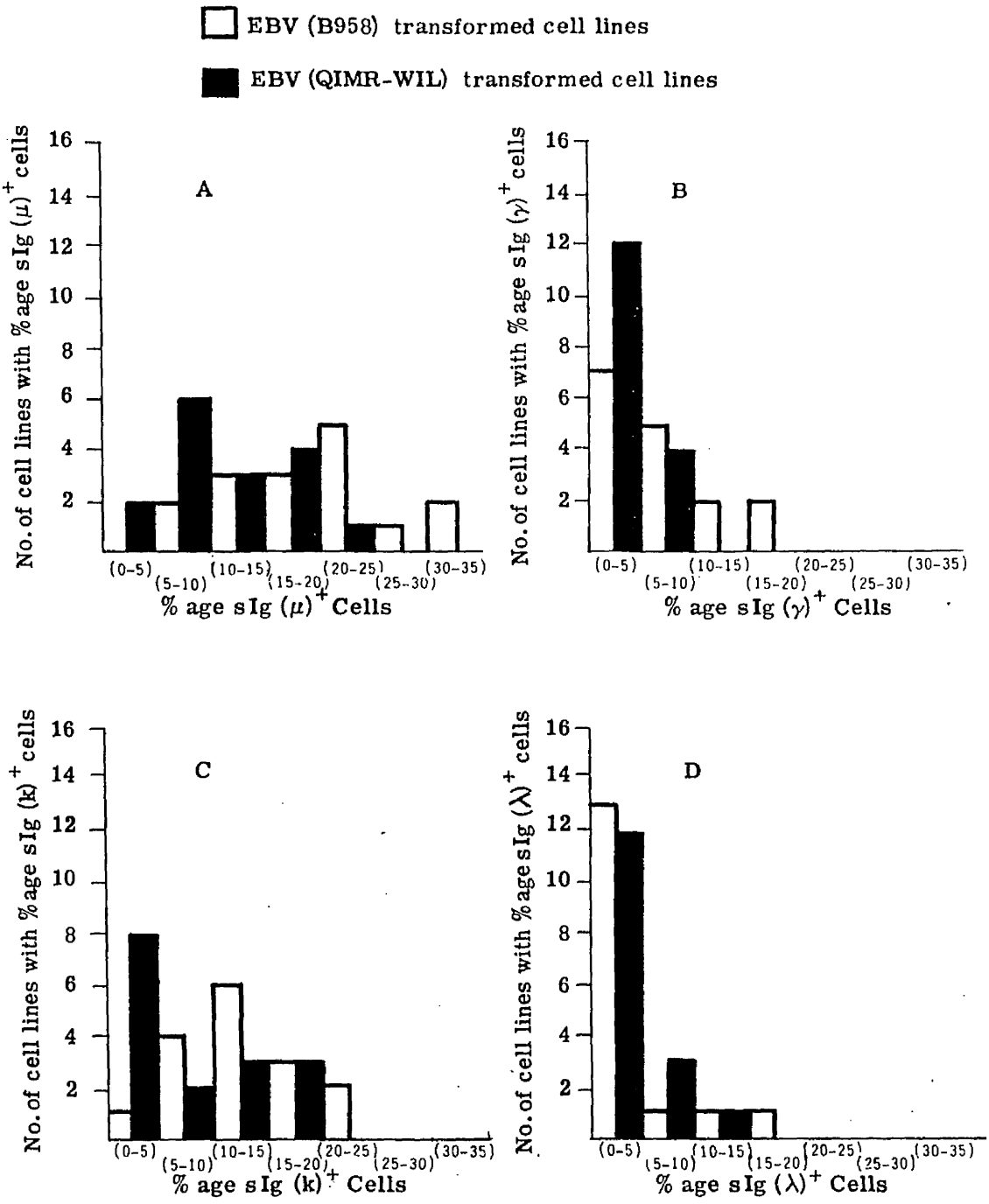
2.2.3. Distribution of sIg^+ Cells Among Cell Lines

Fig. 18 : A = Cell lines with $sIg(\mu)^+$ cells
B = " " " $sIg(\gamma)^+$ "
C = " " " $sIg(k)^+$ "
D = " " " $sIg(\lambda)^+$ "

	No. of cell lines with %age sIg^+ cells detected by Ig type for							
	B9 transformed				QW transformed			
	μ	γ	k	λ	μ	γ	k	λ
Range : 0 - 5		7	1	13	2	12	8	12
" : 5 - 10	2	5	4	1	6	4	2	3
" :10 - 15	3	2	6	1	3		3	1
" :15 - 20	3	2	3	1	4		3	
" :20 - 25	5		2		1			
" :25 - 30	1							
" :30 - 35	2							

The numbers of cell lines with $sIg(\mu)^+$ and $sIg(k)^+$ cells, were distributed over a wider range than those with $sIg(\gamma)^+$ and $sIg(\lambda)^+$ cells respectively, among both B9 and QW transformed cell lines. The numbers of cell lines with $sIg(\mu)^+$, $sIg(\gamma)^+$, and $sIg(k)^+$ cells among B9 transformed cell lines, were distributed over a wider range than those among QW transformed cell lines. However, the ranges of cell lines with $sIg(\lambda)^+$ cells, approximated equality among B9 and QW transformed cell lines.

Fig. 18 Frequency distribution of sIg^+ cells among LCLs



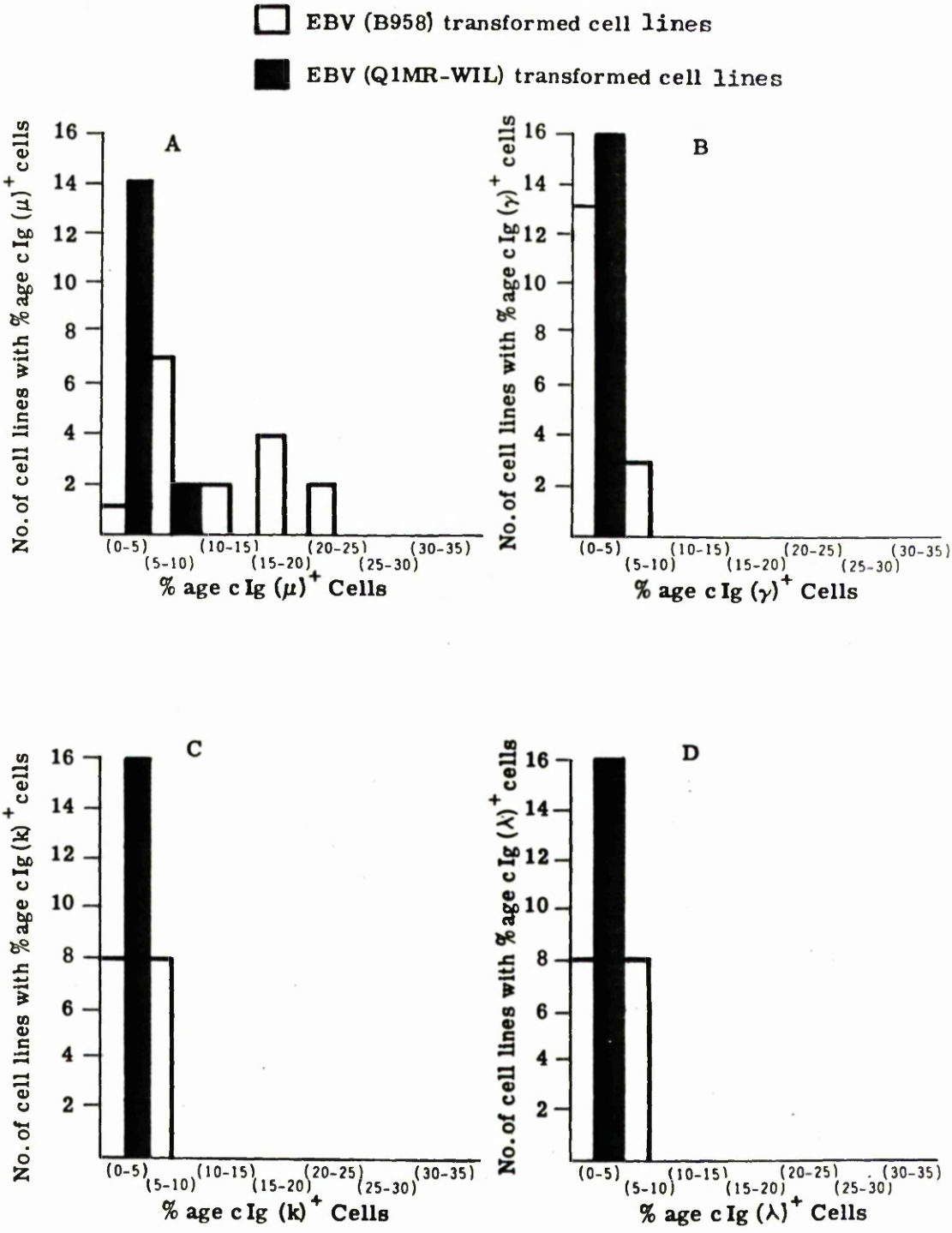
2.2.4. Distribution of cIg^+ Cells Among Cell Lines

Fig. 19 : A = Cell lines with $cIg(\mu)^+$ cells
B = " " " $cIg(\gamma)^+$ "
C = " " " $cIg(k)^+$ "
D + " " " $cIg(\lambda)^+$ "

	No. of cell lines with %age cIg^+ cells detected by Ig type for							
	B9 transformed				QW transformed			
	μ	γ	k	λ	μ	γ	k	λ
Range : 0 - 5	1	13	8	8	14	16	16	16
" : 5 -10	7	3	8	8	2			
" :10 -15	2							
" :15 -20	4							
" :20 -25	2							
" :25 -30								
" :30 -35								

The numbers of cell lines with $cIg(\mu)^+$ cells were distributed over a wider range than those with $cIg(\gamma)^+$ cells, among B9 and QW transformed cell lines. However, the numbers of cell lines with $cIg(k)^+$ and $cIg(\lambda)^+$ cells, were confined to similar ranges among both B9 and QW transformed cell lines.

Fig. 19 Frequency distribution of cIg$^+$ cells among LCLs



2.3. Producer Cell Lines Among Long-term Cultures

2.3.1. Introduction

B9 or QW transformed : -EL77, -LOC78, -IM77 and B9 transformed; B9RK77, B9ELB78 cell lines, were maintained in culture for 18-20 months after the establishment of the transformed state. Using anti-EBV antiserum, F_4-Esther, the percentage $(EA)^+$ cells was determined for each cell line (Methods, 9.3.3). The percentages sIg and cIg $(\mu)^+$ cells were also determined (Methods, 9.3.1 and 9.3.2). The three parameters were then compared as illustrated in Fig. 21.

2.3.2. Discussion

-IM77, B9ELB78 and QWLOC78 cell lines which were $(EA)^-$ 3-5 months after establishment, were found to be $(EA)^+$, 18-20 months after establishment.

The percentage $cIg(\mu)^+$ cells in B9ELB78 cell population was found to be higher among long-term (21.3%) than among short-term (8.3%) cultures. The percentages $sIg(\mu)^+$ cells were found to be similar among long-term (20.8%) and short-term (23.2%) cultures. The corresponding QW transformed cell line, QWELB78, was subjected to the direct IF test to detect the percentages $sIg(\mu)^+$ and $cIg(\mu)^+$ cells. QWELB78 was found to have increased percentages of both sIg and cIg $(\mu)^+$ cells. 5 months after establishment, the percent sIg and cIg $(\mu)^+$ cells were 16.9 and 2.7, but 36.6 and 26.0 respectively, 20 months after establishment.

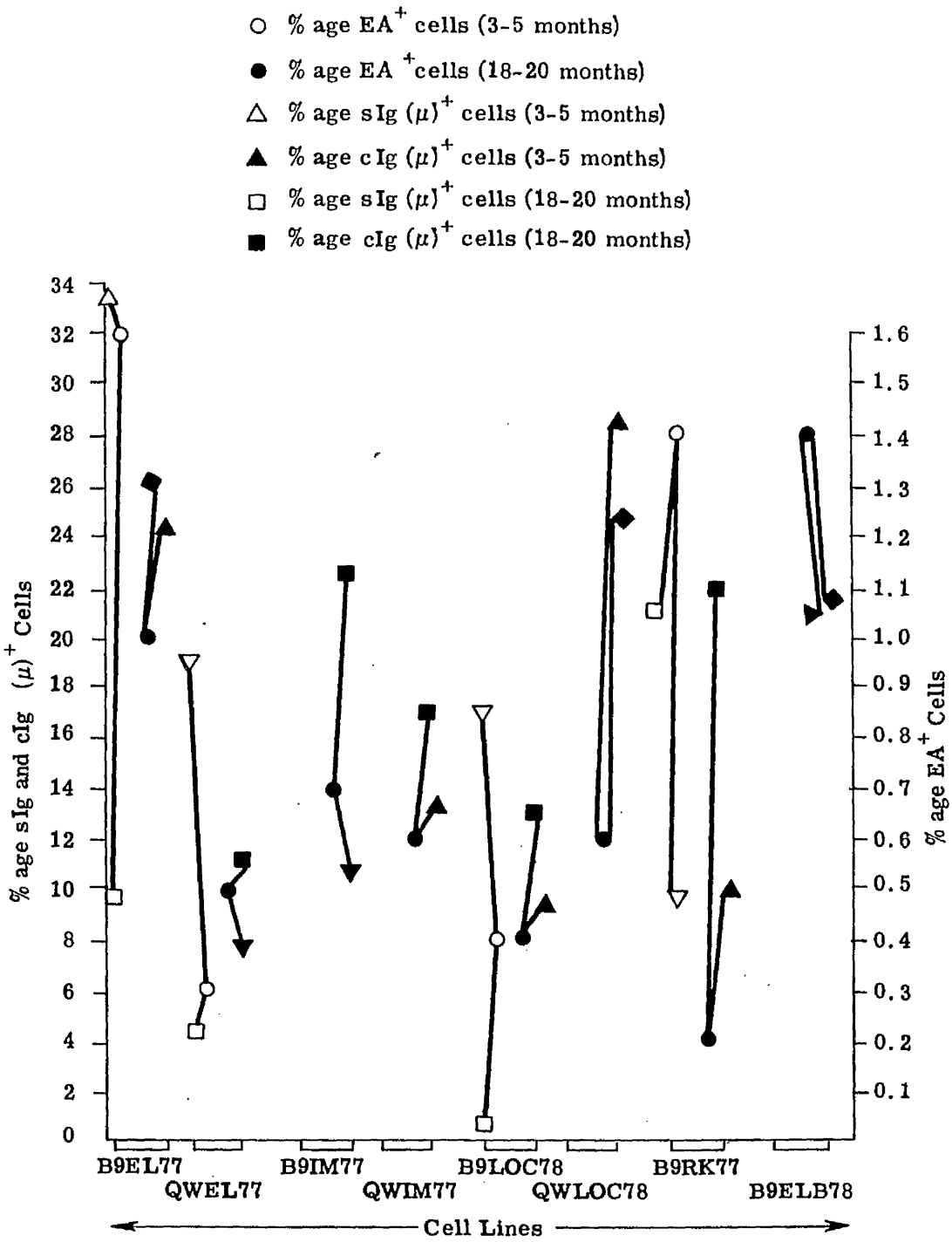
B9 and QW transformed cell lines -IM77 and -LOC78 similarly had higher percentages of $cIg(\mu)^+$ cells among long-term, than among short-term cultures. The percentages $sIg(\mu)^+$ cells between short-term and long-term cultures varied as indicated below :-

Cell Line	% Ig^+ Cells after Time of Establishment			
	3-5 months		18-20 months	
	$sIg(\mu)^+$	$cIg(\mu)^+$	$sIg(\mu)^+$	$cIg(\mu)^+$
B9IM77	20.7	9.1	10.6	22.3
QWIM77	7.4	4.9	13.0	16.8
B9LOC78	16.8	0.4	9.0	13.2
QWLOC78	12.9	3.8	28.0	24.6

Among long-term cultures of -IM77, B9ELB78 and QWLOC78 populations of EA(D) synthesizing cells were selected for continual proliferation. Among long-term cultures of -IM77, -LOC78 and -ELB78, populations of cells with high percentages of $cIg(\mu)^+$ cells were selected for continual proliferation.

The percentages $(EA)^+$ cells among B9LOC78 and QWEL77 were similar between 3-5 and 18-20 month-old cultures. The percentages $(EA)^+$ cells among B9EL77 and B9RK77 populations were found to be lower among 20 month-old than among 5 month-old cultures.

Fig. 21 EA and Ig synthesis in producer cells



The percentages cIg$(\mu)^+$ cells were higher among long-term than among short-term cultures, for B9 and QW transformed -EL77. On the other hand, the sIg$(\mu)^+$ cells varied between increased and decreased percentages in long-term and short-term cultures, as indicated in Fig. 21. The percentages sIg and cIg $(\mu)^+$ cells among B9RK77 populations, were found to be the same in long-term and short-term cultures (Fig. 21). The corresponding QW transformed cell line, QWRK77 was subjected to the direct IF test for the detection of cIg and sIg $(\mu)^+$ cells. Among QWRK77 cell population, the percent sIg$(\mu)^+$ and cIg$(\mu)^+$ cells in short-term culture were 15.7 and 6.6, but 35.6 and 13.4 in long-term cultures, suggesting that, QWRK77 had increased percentages of cIg and sIg $(\mu)^+$ cells.

While the percent sIg$(\mu)^+$ cells fluctuated between increased and decreased percentages, the percentages cIg$(\mu)^+$ cells were higher among long-term than among short-term cultures for both B9 and QW transformed cell lines. Long-term cultivations of EBV-transformed HUCLs therefore had increased percentages of Ig synthesizing cells, irrespective of the viral strain which induced transformation.

2.4. Summary.

Populations of EBV-transformed cells which were $(EA)^-$ in short-term cultures, were found to be $(EA)^+$ in long-term cultures. Among the populations of cells, the percentages of cIg$(\mu)^+$ cells were found to be higher in

long-term than in short-term cultures. On the other hand, sIg(μ)$^+$ cells varied between increased and decreased percentages in long-term and short-term cultures. The possibility therefore existed that, among cultures of EBV-transformed cells, Ig synthesis was a consequence of EBV-associated antigenic stimulation.

Ig, EA and other translational products of EBV-transformed cells have been found to occur at specific stages of the cell cycle (Fig. 22). EBV-transformed cells in culture can therefore be used to elucidate the processes of gene expression in B-cells, with or without antigenic stimulation (Fig. 22).

Fig. 22. References

[1]Slovin et al., (1980)

[2]Rickenson, personal communication

[3]Hampar et al., (1974)

[4]Slovin et al., (1980)

[5]Hampar et al., (1974)

[6]Silvestre et al., (1974)

[7]Hampar et al., (1974)

[8]Takahashi et al., (1969)

[8]Buell et al., (1969)

[9]Thorley-Lawson et al., (1978)

[10]Gergely et al., (1971)

[11]zur Hausen et al., (1967)

[11]Hampar et al., (1974)

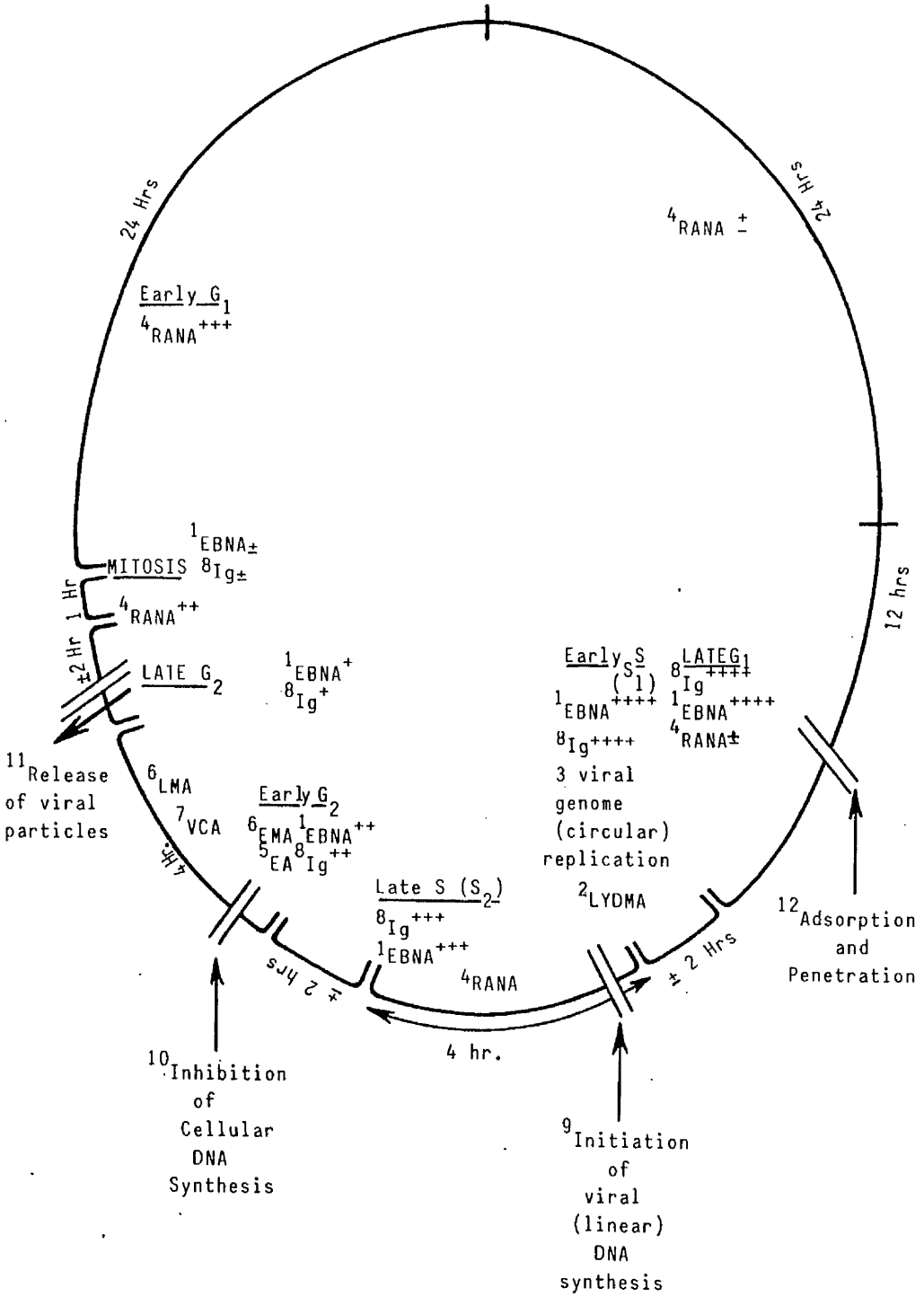
[12]Thorley-Lawson et al., (1978)

Key :-

$\pm$ = barely detectable

++++ = maximal production

Fig. 22 Some translational products of the EBV transformed cell



Chapter 3

The development of Ig(k) dominance

and

The Loss of Ig(μ) dominance

among

Lymphocyte Populations

3.1. Immunoglobulin Production in Long-term Cultures

3.1.1. Introduction

Pairs of B9 or QW infected cell lines; -IM77, -ELB78, -SD77 and -RK77, were maintained in culture over a period of 20 months after establishment. Cells were taken from 2 day-old cultures and the viabilities of the populations determined by the Trypan blue exclusion test. Cultures which were >90% viable, were labelled biosynthetically with ^{35}S-methionine (B9 and QW -RK77) or ^{3}H-leucine (B9 and QW -IM77, -ELB78 and -SD77) with 100 μCi/4 x 10^6 cells for each cell line (Methods, 12.1). The labelled intracellular and secreted proteins were collected. The Ig proteins were precipitated from the samples with RAH antisera against μ (Table 3, Methods), γ, k and λ (Miles) classes and subclasses with GAR IgG as described in Methods; 12.2., 4., and 7. Reduced samples (30 μl/ sample well) were electrophoresed (Methods : 13.1 - 4), fluorographs prepared and processed as described in Methods; 13.5., and 6. All fluorographs were exposed for 42 (^{3}H-leucine label) or 21 (^{35}S-methionine label) days. The data is represented in Figs. 23 - 26.

Abbreviations :-

A/S = RAH antibody type

T/N = Track number; except in Fig. 23 (Analysis of of 'QWELB78),

odd Nos. = intracellular (IN) Ig proteins

even Nos. = secreted (SD) Ig proteins

X in Fig. 25 = L(k) precipitated from Bec-11 cells.
H,L ( ) = Heavy or light chain type.

3.1.2. Synthesis of Ig(H) chains in Long-term cultures

20 month-old cultures synthesized and secreted Ig(μ) among both B9 and QW transformed cell lines :-

-ELB78 : Fig. 23; T/Ns 1 and 5
-RK77 : Fig. 24; T/Ns 1 and 2
-SD77 : Fig. 25; T/Ns 1 and 2
-IM77 : Fig. 26; T/Ns 1 and 2

On the other hand, Ig(γ) was not detectable among 20 month-old cultures of B9 and QW transformed cell lines :-

-ELB78 : Fig. 23; T/Ns 2 and 6
-RK77 : Fig. 24; T/Ns 3 and 4
-SD77 : Fig. 25; T/Ns 3 and 4
-IM77 : Fig. 26; T/Ns 3 and 4

The rate of turnover for Ig(μ) synthesis was greater among B9 transformed cell lines; B9ELB78, B9RK77, B9SD77 and B9IM77. This observation was evidenced by the much more intense sharper bands which were observed among intracellular and secreted μ chains of the B9 transformed than among the corresponding QW transformed cell lines (Figs. 23 - 26).

Fig. 23 Analysis of Ig proteins of QWELB78 and B9ELB78

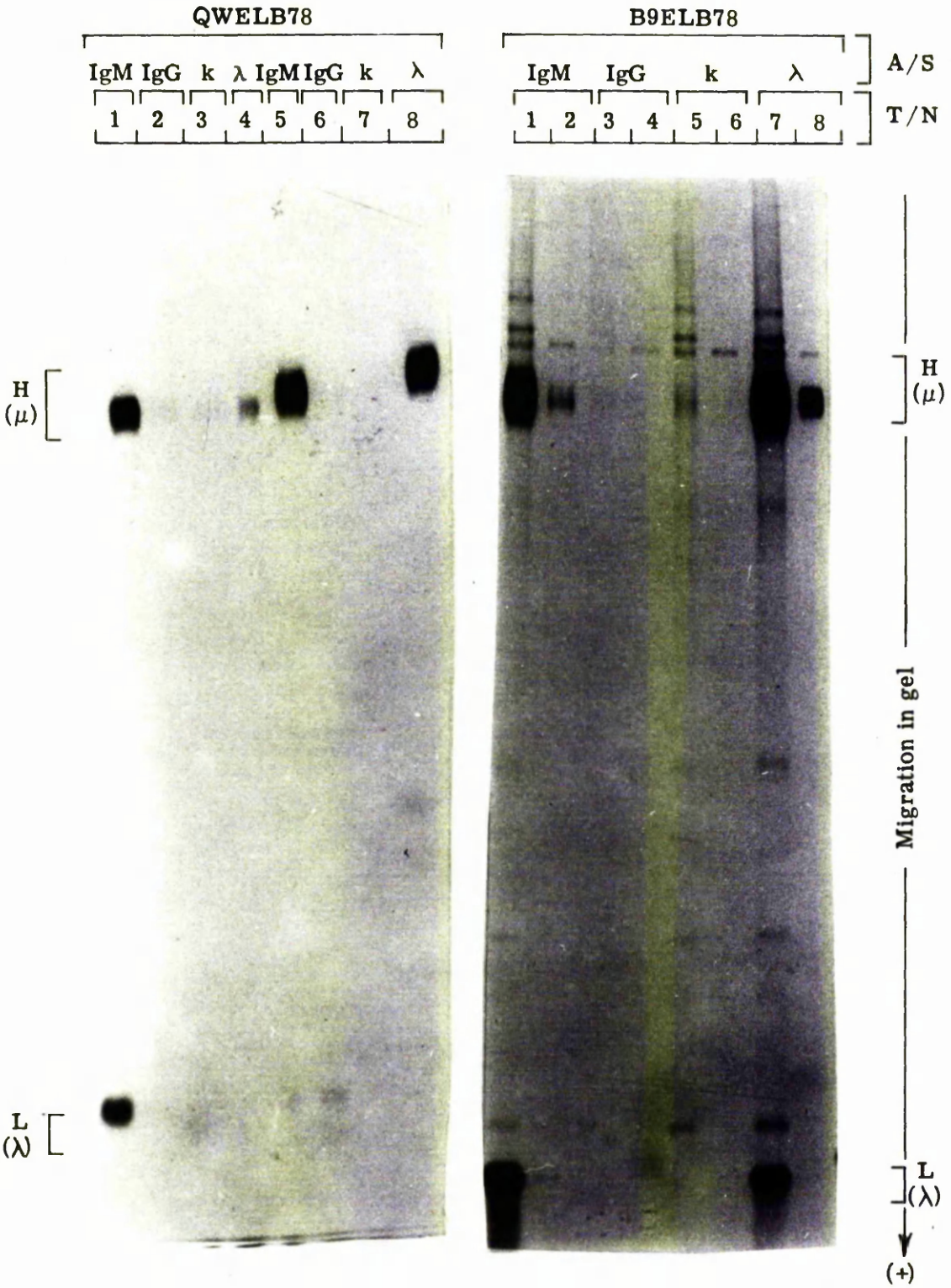


Fig. 24 Analysis of Ig proteins of QWRK77 and B9RK77

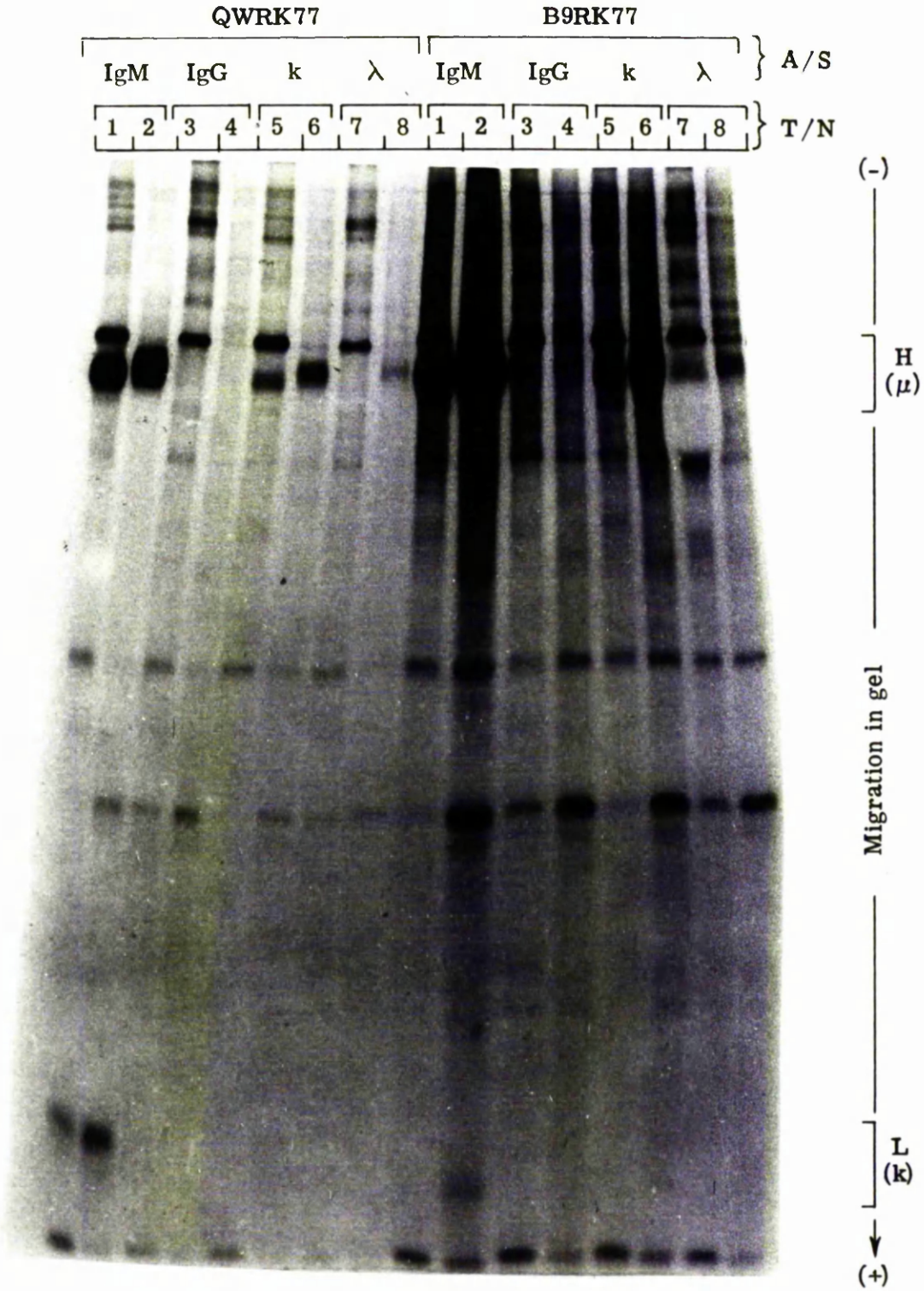


Fig. 25 Analysis of Ig proteins of QWSD77 and B9SD77

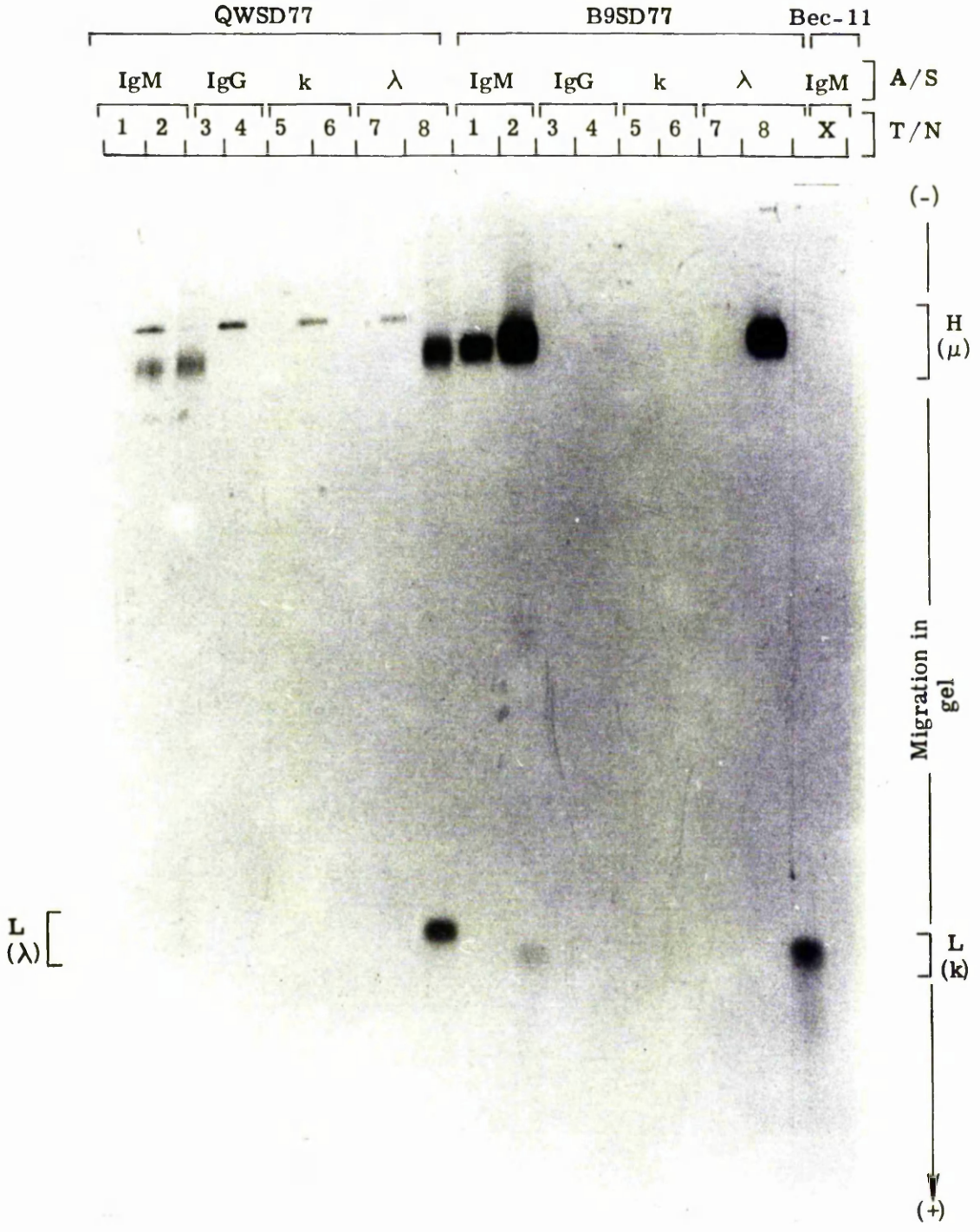
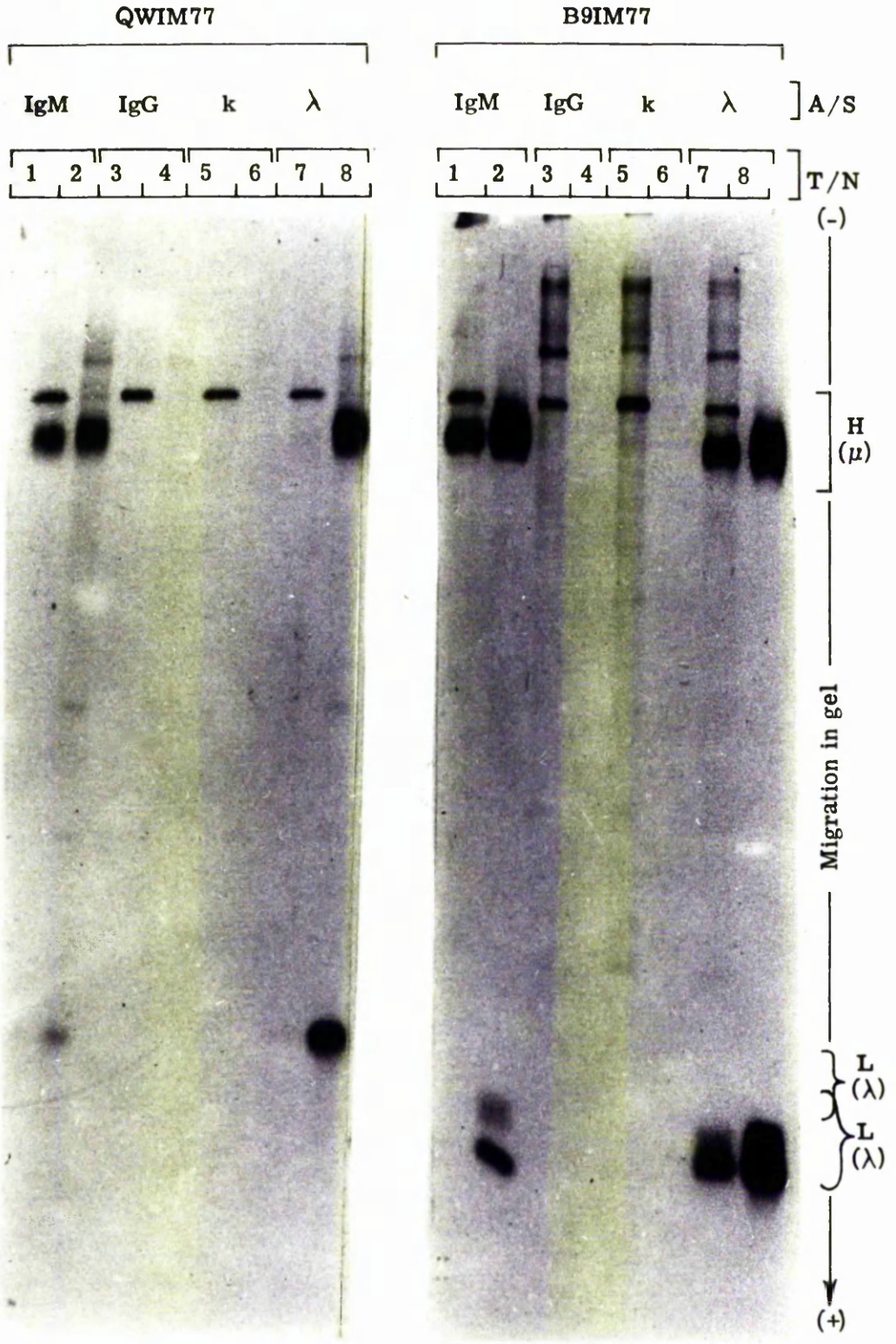


Fig.26 Analysis of Ig proteins of QWIM77 and B9IM77



3.1.3. Synthesis of Ig(L) Chains in Long-term Cultures

One pair of cell lines in long-term cultures (-RK77), was found to synthesize L(k) but not L(λ) :-

Fig. 24 : T/Ns 1, 2, 5 and 6

On the other hand, B9 and QW transformed cell lines -SD77, -IM77 and -ELB78 synthesized L(λ) but not L(k) as indicated below :-

QWELB78 : Fig. 23; T/Ns 1 and 4

B9ELB78 : Fig. 23; T/Ns 1 and 7

-SD77 : Fig. 25; T/Ns 2 and 8

QWIM77 : Fig. 26; T/Ns 2 and 8

B9IM77 : Fig. 26; T/Ns 2, 7 and 8.

The long-term cultures of B9 and QW transformed cell lines; -SD77, -IM77, -RK77, and -ELB78 were subjected to the direct IF test for the detection of cIg and sIg, $(k)^+$ and $(\lambda)^+$ cells (Methods, 9.3.1 and 9.3.2).

While Ig(k) was detected, Ig(λ) was not detected in the cytoplasms or on the surfaces of B9RK77 and QWRK77 cells. On the other hand, Ig(λ) was detected, but Ig(k) was not detected in the cytoplasms or on the surfaces of; -SD77, -IM77, and -ELB78 cells. Among the above pairs of cell lines, there was therefore an imbalance between $Ig(k)^+$ and $Ig(\lambda)^+$ cells in favour of $Ig(\lambda)^+$ cells.

Within pairs of cell lines, similar light chains were synthesized in long-term cultures. There was undoubtedly a selection of Ig(λ) synthesizing cells above Ig(k) synthesizing cells among B9 and QW transformed -SD77, -ELB78 and -IM77. Barandun et al., (1976) similarly

found that, among pairs of maternal and umbilical cord blood samples, there was an imbalance of the equality of Ig(k) and Ig(λ) in favour of Ig(λ). It is therefore probable that among certain neonatal lymphoid cell populations, there is a selection of Ig(λ) above Ig(k) light chain type.

3.2. Distribution of Ig Synthesizing Cells Among Lymphocyte Populations

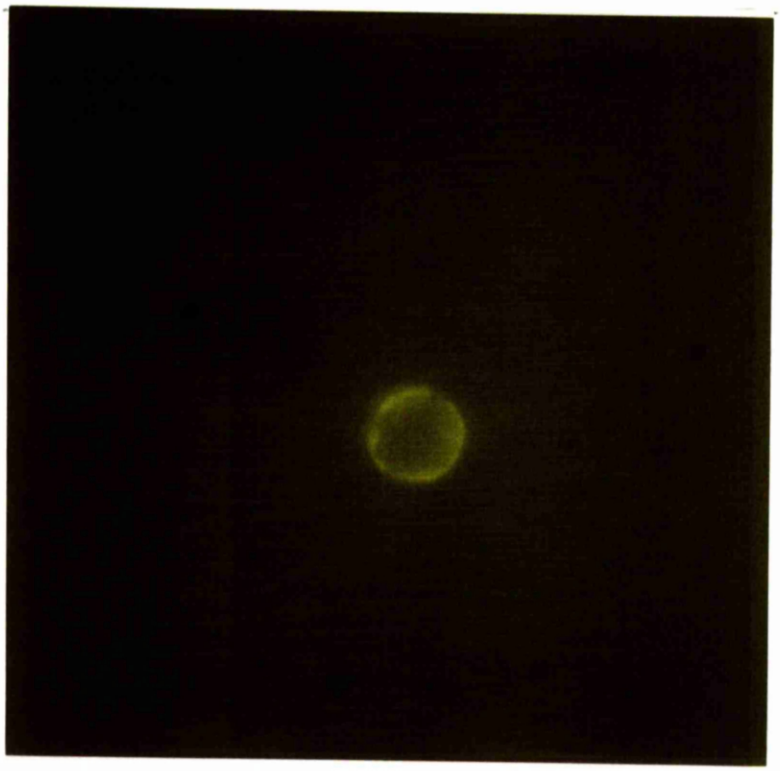
3.2.1. Introduction

Samples of blood were collected from human umbilical cords, or the peripheral blood of subjects of varying ages (in most cases, 3 samples for each age study). Heparinized blood (20 i.u./ml) was subjected to Ficoll-hypaque density gradient centrifugation (Method, 5.3) and mononuclear cells collected. Using FITC-conjugated RAH antisera which had been raised against μ, γ, k and λ heavy and light chain types (Behring), the mononuclear cells were subjected to the direct IF test (Methods, 9.3.1).

Mononuclear cell populations were collected from the spleens and livers of Balb/c mice of varying ages, by Ficoll-hypaque density gradient centrifugation. The viabilities of the cell suspensions were determined by the Trypan blue exclusion test (Methods, 2.2). The densities of the suspensions were determined with white cell counting fluid (Methods, 5.3.1). The cells were then subjected to the indirect methods of IF (Methods, 9.5). The patterns of fluorescence of sIg^+ cells among untransformed human lymphocyte populations was similar to Fig. 16. The pattern of fluorescence of sIg^+ cells among murine lymphocyte populations is displayed in Fig. 26A.

The percentages of sIg $(k)^+$ and $(\lambda)^+$ cells among murine lymphocyte populations as well as the percentages

Fig. 26A. Murine spleen lymphocyte (sIg^+) : prepared by indirect IF (Methods, 9.5) and photographed with Ektachrome 400. (Magnification x 100).



sIg $(\mu)^+$ $(\gamma)^+$ $(k)^+$ and $(\lambda)^+$ cells among human lymphocyte populations were determined.

3.2.2. Distribution of Ig Classes Among Normal Human Lymphocyte Populations

The percent sIg$(\mu)^+$ and sIg$(\gamma)^+$ cells among untransformed HUCL populations were found to be 21.3 and 6.5, but 15.1 and 12.5 respectively among untransformed lymphocyte populations which had been obtained from young adults (20-25 yrs).

The percentage sIg$(\mu)^+$ cells was found to decrease with age (Fig. 27). Between 0 and 10 yrs, there was gross fluctuation, with increases and decreases among Ig(μ) synthesizing cells. This fluctuation probably reflected the post-natal increase of Ig(μ) synthesizing cells, as well as the response of Ig synthesizing cell populations to antigenic stimulation. Between 10 and 20 years, Ig$(\mu)^+$ cells remained at similar numbers, but higher than the numbers of Ig(γ) synthesizing cells. Ig$(\mu)^+$ cells approximated equality with Ig(γ) synthesizing cells between 20 and 30 years.

3.2.3. Distribution of Ig Subclasses Among Murine Lymphocyte Populations

The percent sIg$(\lambda)^+$ and sIg$(k)^+$ cells at birth (>24 hrs old), were 2.81 and 2.26 respectively, among lymphocyte populations which had been obtained from the spleens of

Balb/c mice. Among lymphocyte populations which had been obtained from the livers of newborn mice, $sIg(\lambda)^+$ cells similarly showed a slight dominance over $sIg(k)^+$ cells. The percent $sIg(\lambda)^+$ cells was found to be 2.3, while the percent $sIg(k)^+$ cells was found to be 2.01.

$Ig(k)^+$ cells increased with age among murine lymphocyte populations (Fig. 28). At birth, the percentage $Ig(k)^+$ and $Ig(\lambda)^+$ cells approximated equality. The percentage $sIg(k)^+$ and $sIg(\lambda)^+$ cells increased between 0 and 7 days after birth. However, there was a much sharper increase in the numbers of $sIg(k)^+$ cells than among $sIg(\lambda)$ cells during this period of time. While $sIg(k)^+$ cells continued to increase after age 7 days, there was no significant increase in the percentage $sIg(\lambda)^+$ cells after age 7 days. The percentage $sIg(\lambda)^+$ cells between 7 days and 15 weeks, approximated equality.

Fig. 27 Distribution of sIg $(\mu)^+$ cells among human lymphocyte populations

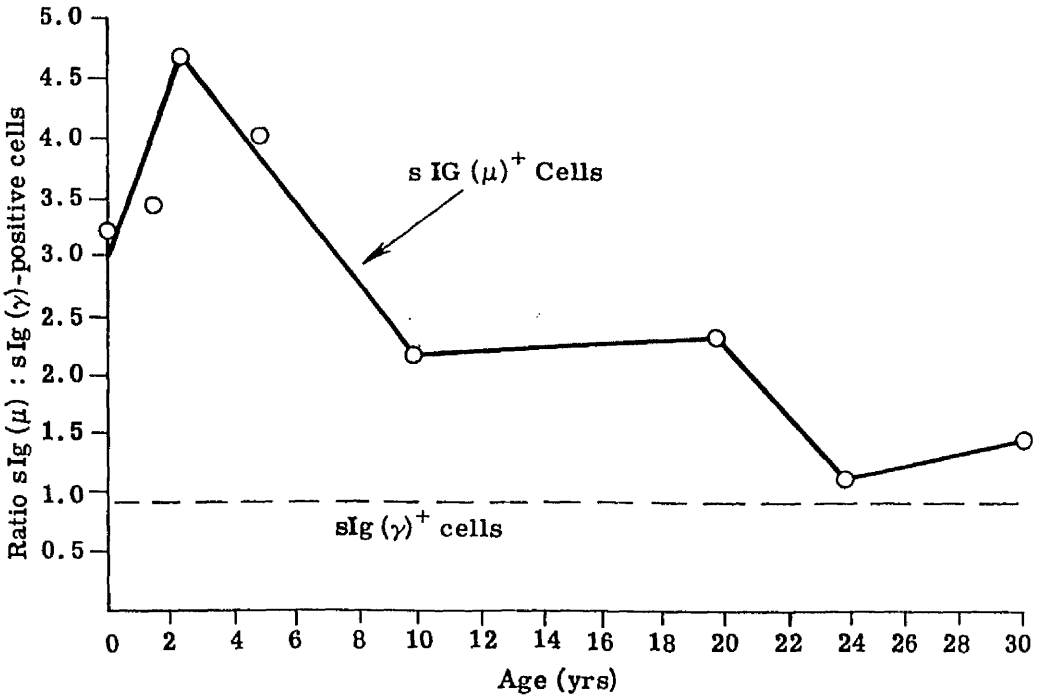
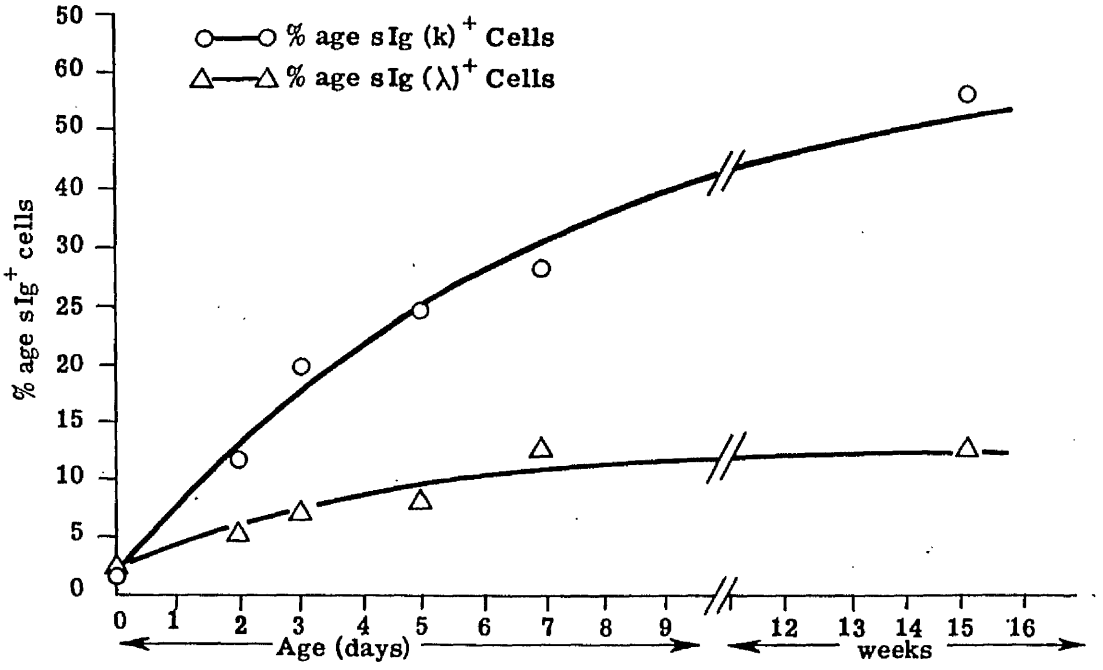


Fig. 28 Distribution of sIg (k) /$(\lambda)^+$ cells among murine lymphocyte populations



3.2.4. Development of Ig(k) Dominance Among Murine and Human Lymphocyte Populations

The ratios of the percentages $sIg(k)^+$ and $sIg(\lambda)^+$ cells were determined among human (Fig. 29) and murine (Fig. 30) lymphocyte populations.

The development of Ig(k) dominance among murine and human lymphocyte populations were found to occur in 3 phases as indicated below :-

humans : phase 1 = 0-5 yrs approx.
phase 2 = 5-20 " "
phase 3 = 20 years onwards.

mice : phase 1 = 0-3 days approx.
phase 2 = 3-9 " "
phase 3 = 9 days onwards.

The response of Ig synthesizing cells to antigenic stimulation among human lymphocyte populations was determined. Blood samples were taken from human donors with various pathological symptoms (Table 3.1), and subjected to the direct IF test (Methods 9.3.1).

While the percentages of $sIg(\mu)^+$ and $sIg(\gamma)^+$ cells fluctuated between ages 0-24 years in response to various pathological conditions, the ratios of $sIg(k)^+$: $sIg(\lambda)^+$ cells existed in 3 distinct phases. These phases were similar to those among "normal" subjects. This suggested that $Ig(k)^+$ cells responded preferentially to antigenic stimulation above $Ig(\lambda)^+$ cells during the developing stages of humans (and most probably mice).

Fig. 29 Distribution of ratios sIg $(k)^+$ and sIg $(\lambda)^+$ cells among human lymphocyte populations

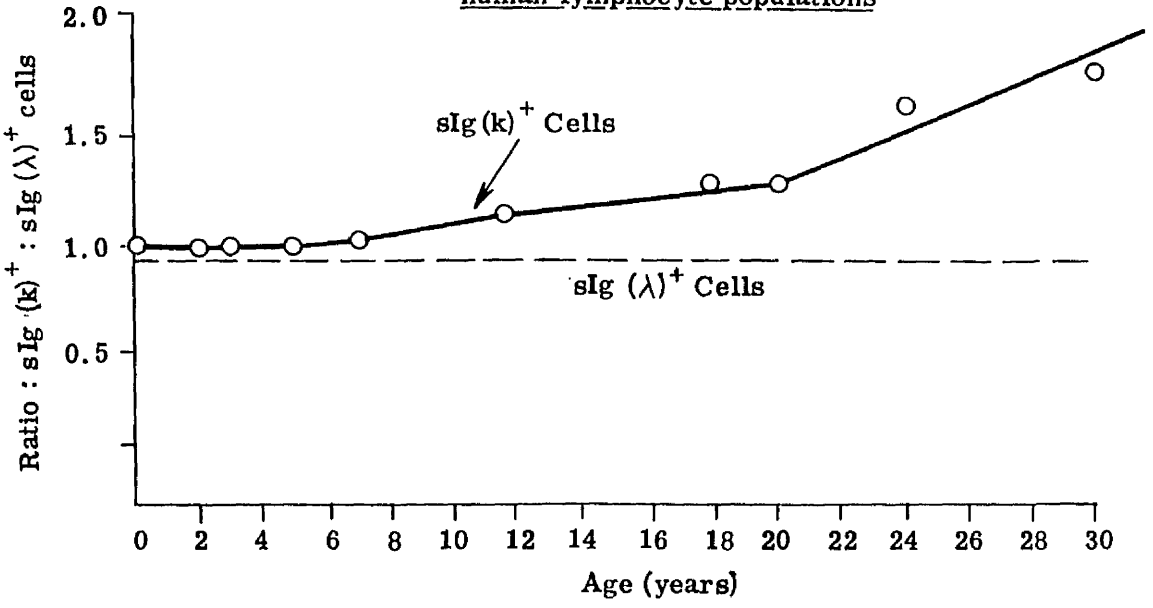


Fig. 30 Distribution of ratios sIg $(k)^+$ and sIg $(\lambda)^+$ cells among murine lymphocyte populations

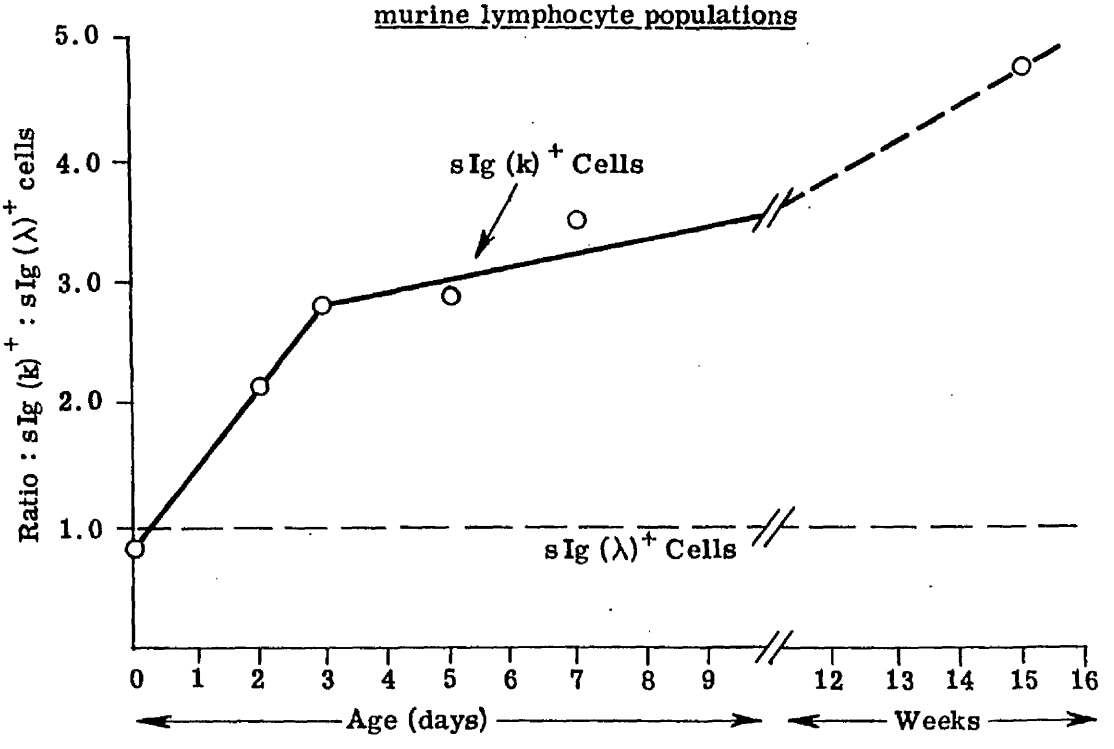


Table 3.1. Ratios of sIg^+ Cells in Relation to Pathology of Donor

Age of Donor (years)	Ratios of sIg^+ Cells $\mu:\gamma(1.0)$	$k:\lambda(1.0)$	Pathological Complaint
0.0	3.3	1.05	HUCLs
1.2	8.0	1.05	Bronchitis
1.5	3.4	1.04	Congenital heart disease
3.0	1.5	1.04	Recurrent chest infections
5.0	4.0	1.04	Epilepsy
7.0	4.8	1.07	Cystic fibrosis
9.5	6.0	1.37	Diabetic
10.0	2.2	1.33	Congenital cardiac failure
20.0	1.9	1.28	Influenza
24.0	1.1	1.60	"

GENERAL DISCUSSION

Lymphoblastoid cell lines (LCLs) have been established from patients with IM and BL (Pagano et al., (1976); Epstein et al., (1965)). LCLs have also been established by the transformation of lymphocytes in vitro by EBV (Katsuki et al., (1975)). The cells which are susceptible to EBV infection, have been shown to bear surface immunoglobulin and have Fc as well as C'3 receptors ($sIg^{+}Fc^{+}$(B)-cells; Jondal et al., (1973)). While BL-derived LCLs have been shown to be sIgM positive (Klein et al., (1968)), other populations of EBV-infected (B)-lymphocytes have been shown to be polyclonal in nature (Rosen et al., (1977)).

Among the 16 pairs of cell lines which had been transformed by EBV B958 and EBV QIMR-WIL, both IgM and IgG were detectable in the cytoplasms as well as on the surfaces of most cell lines which had been maintained in culture for 3-5 months after the establishment of the transformed state had been determined. Katsuki et al., (1977) found that the frequency of EBV-transformed HUCL populations and sIgM positive cells not only correlated, but had similar values. They suggested that sIgM positive cells were the target cells for EBV-induced transformation among HUCL populations. However, in the system which was employed by Katsuki et al., (1977), the only monospecific antiserum which had been used, was directed against Ig(μ) heavy chain type. Yamamoto et al., (1976) established 22 EBV-infected LCLs from tonsillar lymphocytes which had been obtained from children of varying ages. They showed that eight of these 22 cell lines produced cytoplasmic IgA.

Steinitz et al., (1980) preselected and successfully EBV-transformed sIg(A) positive lymphocytes from HPB and lymphocyte populations which had been obtained from NPC patients. These findings strongly suggest that sIg positive cells, other than sIgM positive cells are indeed susceptible to EBV-induced transformation. It is a probability that all $sIg^{+}Fc^{+}$ B-cells are susceptible to EBV-induced transformation. The populations of B-lymphocytes which are susceptible to EBV-induced transformation in vitro are most probably dependent on the population of Ig synthesizing cells in vivo (Steel et al., (1977)).

Katsuki et al., (1975) found that IgG and IgM could be detected in the cytoplasms of newly transformed EBV-infected HUCL populations of cells. However, they found that sIgM but not sIgG could be detected among populations of EBV-transformed HUCLs. The failure of Katsuki et al., (1975) to detect sIgG positive cells, could have been due to the age (4-5 months old) of the cultures which had been tested for the detection of sIgG positive cells. Secondly, a smaller number of cell lines (6 pairs) were investigated by Katsuki et al., (1975).

Among long-term cultures of EBV (B958) and EBV (QIMR-WIL) transformed cells, IgM but not IgG synthesizing cells could be detected among HUCL populations. This finding agrees favourably with those of Evans et al., (1974) and Steel et al., (1977). The percentages of IgM synthesizing cells among normal human lymphocyte populations, were found to vary with age. At birth, the

ratio of IgM : IgG synthesizing cells was 3.3 : 1.0; 4.8 : 1.0 at age 2½ years, but decreased to 2.2 : 1.0 at age 10 years. Between the ages of 10 and 20 years, the ratios of the percentages of IgM : IgG synthesizing cells were 2.2 : 1.0 and 2.3 : 1.0 respectively. The percentages of IgM synthesizing cells approximated equality with IgG synthesizing cells after 20 years of age. The percentages of IgM positive cells therefore exceeded the percentages of IgG synthesizing cells among lymphocyte populations which had been obtained from infants. Among lymphocyte populations which had been obtained from adolescents, there was a fractional difference between the percentages of IgM and IgG synthesizing cells, in favour of IgM synthesizing cells. James et al., (1974) similarly found that in children (<10 years old) the percentages of lymphocytes which bore sIgM were greater than those which bore sIgG. Among lymphocyte populations which had been obtained from adults, James et al., (1974) found that the percentages of lymphocytes which bore IgG and IgM, approximated equality.

The occurrence of high titre heterophile sheep cell agglutinins which occur during IM has been well documented. All available evidence indicates that these antibodies are exclusively 19S (IgM) in nature (Wollheim et al., (1966)). The fractional difference between IgG and IgM synthesizing cells among adolescents could possibly account for the proliferative response of IgM synthesizing cells among adolescents who contract IM.

The development of Ig(k) dominance was found to exist in three phases among lymphocyte populations which had been obtained from human donors. The ratio of the percentages of Ig(k) : Ig(λ) synthesizing cells was found to be constant at equality between the ages of 0 and 5 years, but increased between 5 and 20 years in favour of Ig(k) synthesizing cells. Ig(k) synthesizing cells maximized after age 20 years. The development of Ig(k) dominance was similarly found to exist in three phases among mouse lymphocyte populations. The ratios of the percentages of Ig(k) : Ig(λ) synthesizing cells were found to vary between approximately 0.9 : 1.0 and 2.9 : 1.0 between 0 and 3 days respectively, but 3.5 : 1.0 at 9 days after birth. There was a further increase in the ratio of Ig(k) : Ig(λ), in favour of Ig(k) synthesizing cells between 9 days and adulthood (15 weeks). Haughton et al., (1978) similarly found that among lymphocyte populations which had been obtained from mouse spleens, the dominance of Ig(k) synthesizing cells, developed as a function of age. They suggested that the predominance of Ig(k) synthesizing cells in adult mice had been established by an antigen-driven clonal expansion of the repertoire of Ig(k) genes which expanded as a function of age. Hood et al., (1967) suggested that there is an evolutionary relatedness between the genes which controlled the synthesis of Ig(k) subclasses among human and mice species. They found that, 60% of the amino acid residues which had been obtained from humans and mice Ig protein subclass Ig(k) constant region proteins, were similar in their sequence distributions. If the

development of the dominance of Ig(k) synthesizing cells (consequently the gene pools which control Ig(k) synthesis) among human lymphocyte populations exist in three phases, it is therefore not surprising that the development of Ig(k) dominance among murine lymphocyte populations (consequently the gene pools which control Ig(k) synthesis), should similarly exist in three phases.

Among lymphocyte populations which had been obtained from donors with various pathological disorders, the ratios of the percentages of IgM : IgG synthesizing cells were found to vary. The ratios of the percentages of Ig(k) : Ig(λ) synthesizing cells among these subjects were found to exist in three phases which were comparable with normal subjects. Among lymphocyte populations which had been obtained from one donor (1 year old) who had bronchitis, the ratio of IgM : IgG synthesizing cells was 8.0 : 1.0. Among lymphocyte populations which had been obtained from one epileptic donor (5 years old), the ratio of the percentages of IgM : IgG synthesizing cells was 4.0 : 1.0. However, the ratios of the percentages of Ig(k) : Ig(λ) synthesizing cells which had been obtained from the subjects at 1 and 5 years were constant at approximately 1.0 : 1.0. The ratios of the percentages of IgM : IgG synthesizing cells among lymphocyte populations which had been obtained from one subject with cystic fibrosis (7 years old), was 4.8 : 1.0, but 2.2 : 1.0 among lymphocyte populations which had been obtained from one subject who had congestive heart failure (10 years old). The ratios of the percentages of

Ig(k) : Ig(λ) synthesizing cells was 1.1 : 1.0, but 1.3 : 1.0 among lymphocyte populations which had been obtained from subjects at 7 and 10 years respectively. Among lymphocyte populations which had been obtained from two subjects (20 and 24 years) who had influenza, the ratios of the percentages of IgM : IgG synthesizing cells were 1.9 : 1.0 and 1.1 : 1.0 respectively. The ratios of the percentages of Ig(k) : Ig(λ) synthesizing cells among subjects at 20 and 24 years, were 1.3 : 1.0 and 1.6 : 1.0 respectively.

These findings would agree favourably with the suggestion of James et al., (1974), that the elevated numbers of lymphocytes bearing IgM early in life, is replaced with age and/or antigenization, by lymphocytes bearing IgG. Secondly, the development of the light chain type kappa dominance among Ig synthesizing cells may also reflect maturation and/or antigenization.

Burkitt's lymphoma is primarily a childhood disease with a peak incidence at age 5 years (Burkitt, (1962)). From the foregone data, it is conceivable that age 5 is crucial in terms of the utilization of the gene pools which direct Ig syntheses among Ig synthesizing cells. This situation could predispose subjects with a depressed immune system (e.g. among malarial subjects) to EBV-induced oncogenesis in vivo.

BL-derived LCLs in culture almost always synthesize IgM heavy chain class (Fialkow et al., (1973); Klein et al., (1968)). The decrease in the preponderance of IgM

synthesizing cells with age, and the association of BL with infants in particular and juveniles in general, obviously contribute to the dominance of IgM synthesizing cells among BL-derived LCLs. The ratio of the percentages of IgM : IgG synthesizing cells among untransformed HUCL populations was found to be 3.3 : 1.0. The ratio of the percentages of IgM : IgG synthesizing cells among EBV (B958) and EBV (WIMR-WIL) transformed cell lines (3-5 months old), were found to be 3.0 : 1.0 and 3.3 : 1.0 respectively. This suggested that IgM synthesizing cells exceeded IgG synthesizing cells among untransformed as well as EBV-transformed HUCLs. Among long-term cultures of EBV (B958) and EBV (WIMR-WIL) transformed cells, IgM, but not IgG synthesizing cells could be detected. The selection of IgM synthesizing cells among long-term cultures of EBV-transformed HUCLs was therefore attributable to the pool size of the IgM synthesizing cells among HUCL populations.

Long-term cultures were found to have higher numbers of producers (for EA synthesis) than the corresponding short-term cultures. While long-term cultures were found to consistently have higher percentages of cytoplasmic IgM positive cells, surface IgM positive cells fluctuated between increased and decreased percentages. Wollheim et al., (1966) suggested that one mechanism for the formation of the heterophile antibody among IM patients could be that EBV shares antigenic determinants with IgM. Tovey et al., (1978) found that anti-IgM antibody could induce the

synthesis of EA in Raji cells. The induction of EA in Raji cells was Ig(μ) specific since antisera to : Ig(k), Ig(λ), IgA, IgE and IgD were not effective in inducing the synthesis of EA. Among long-term cultures of EBV-transformed HUCLs, Ig(μ) was not only detectable on the surfaces and in the cytoplasms of transformed cells, but was also secreted into the extracellular growth medium as detected by SDS-polyacrylamide gel-electrophoresis. It is therefore highly probable that the released Ig(μ) chains induced the synthesis of EA among established cultures of EBV-transformed cells. Furthermore, one cell line which had a decrease percentage of EA synthesizing cells during long-term cultivation, showed no difference in the percentages of cytoplasmic or surface Ig positive cells between short-term and long-term cultures.

The numbers of IgM, IgG, Ig(k) and Ig(λ) positive (both sIg and cIg positive) cells among EBV (B958) transformed cells, exceeded the corresponding numbers of Ig positive cells among EBV (QIMR-WIL) transformed cells. Similar findings were observed by Katsuki et al., (1975) - these workers did not observe or compare the frequency of Ig(λ) synthesizing cells among EBV (B958) or EBV (QIMR-WIL) transformed cells. These findings strongly implicate the possibility that EBV (B958) transformed cells had higher percentages of differentiated $sIg^{+}Fc^{+}$ B-cells, than EBV (QIMR-WIL) transformed cells.

The time lapse between infection and establishment of the transformed state was found to be longer among EBV (QIMR-WIL) infected, than among EBV (B958) infected cultures of cells. The transforming efficiency of EBV B958 was found to be higher than the transforming efficiency of EBV (QIMR-WIL). The higher transforming efficiency of EBV B958 could account for the shorter time lapse between infection and establishment of the transformed state among EBV (B958) infected cultures of cells. However, the possibility cannot be eliminated that undifferentiated B-cells are less subject to EBV-induced transformation. Consequently, EBV (QIMR-WIL) infected cells would require a much longer period of time for the establishment of the transformed state than EBV (B958) infected cells.

The application of these studies to the study of the control of the immune system cannot be overlooked. If EBV (B958) and EBV (QIMR-WIL) transformed cells represent different percentages of differentiated B-cells it is not difficult to envisage that different Ig responses should be observed during mimicry of the immune response *in vitro* among these two groups of cells. Kishimoto *et al.*, (1978) have described the mimicry of the immune response *in vitro* among established LCLs of various origins. If different Ig responses are observed following the induction of EBV (B958) and EBV (QIMR-WIL) transformed cells after T-cell stimulation (Kishimoto *et al.*, (1978)), this would undoubtedly present a system for the study of B-cell

differentiation *in vitro*. Since Ig and non-Ig synthesizing, EBV-transformed cells *in vitro* represent Ig and non-Ig synthesizing cells *in vivo* (Steel *et al.*, (1977)), the studies could then be related to B-cell differentiation *in vivo*.

Work is in progress to identify the Major Histocompatibility Complex alloantigenic markers which are borne on the pairs of EBV (B958) and EBV (QIMR-WIL) transformed cells.

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ADDENDUM

FACTORS GOVERNING THE EXPRESSION OF EBV-ASSOCIATED DISEASES

There is no doubt that the special availability of appropriate target cells, host immunological capacity and genetically determined characteristics are factors which govern the type of symptoms which are expressed among individuals who contract EBV.

Burkitt's Lymphoma

BL is commonly observed in areas of Africa (Burkitt, 1958), New Guinea (Ten Seldam et al., 1966), and less commonly in the United States (Ziegler et al., 1976) and Europe (Bornkamm et al 1976). Although sex ratio and age-grouping show remarkable similarity between New Guinean and African BL, there has been no distinct geographical and climatic limitations observed in New Guinean BL as seen in African BL. It therefore seems highly probable that an environmental factor could be associated with African BL to allow this distinct geographical and climatic distribution to be displayed.

The fact that BL is most common in malarial endemic areas of Africa would implicate malaria as the contributory environmental factor which is associated with African BL. Malaria is a powerful stimulator of the reticuloendothelial system and constant onslaught on the cells of this system in malarial-endemic areas might provide the correct host environment to bring about malignant transformation. Children with acute malaria have been shown to have a diminished antibody response to certain antigens. Greenwood et al., 1972 found that among children in Gambia in which malarial prophylaxis was being carried out, there was a greater antibody response to tetanus toxoid than in children in which malarial prophylaxis was not being carried out. Persistent malaria could therefore act as a cofactor by stimulating and maintaining a continuing large supply of lymphoid cells, especially liable to undergo malignant change in response to infection by the virus.

Where BL is endemic in Africa, almost the entire population is infected by EBV at an early age (Henle et al., 1969) and hyper-endemic malaria affects over 50% of young children. Yet, only small numbers of these doubly infected individuals develop the tumour. It seems likely, that, in addition to an environmental factor, a genetic predisposition to BL could also exist. Fialkow

et al., 1970; 1973 found that BL is of monoclonal origin. However, there is as yet, no evidence which would point to a genetic predisposition to BL as observerd among NPC subjects.

Infectious Mononucleosis

IM only occur in individuals without antibodies to EBV, since all pre-illness sera available by chance or by design from prospective studies of IM proved to be uniformly devoid of all antibodies to EBV (Henle et al., 1973). The disease is rare in persons over 35 years old i.e., in age ranges in which over 80% of individuals possess antibodies to EBV (Henle et al., 1966). Yoshida et al., 1980 found that antibody titres to VCA among randomly selected infants (0 - 3 years) and children showed that 35% of infants had acquired antibodies to VCA. All antibodies to VCA appeared after 7 months althouugh none developed the symptoms of IM. IM is primarily associated with adolescents and young adults because of the large amounts of viral particles obtained on infecion during this age group.

Henle et al., 1970 found that there is a low frequency of EBV infections in children from middle and upper socio-economic status, consequently a large number of individuals from these groups reach adolescence and early adulthood without contracting the virus. IM however, is rare among Africans in young adolescents and young adults. Henle et al., 1969 found that sera which had been taken from African children who served as control groups (without African BL) showed that EBV is widespread in Africa as elsewhere in the world. Primary infections occur in Africa more often and early in life. As a result, few individuals reach adolescence and early adulthood without detectable antibodies to EBV. This explains the rarity of IM among Africans in these age groups.

IM is a self-limiting disease - T-cells which specifically destroy EBNA-positive cells from EBV-transformed LCL in vitro have been demonstrated by ^{51}Cr-release technique among circulating lymphocytes of IM patients during the acute phase of the illness (Svedmyr et al., 1975; Royston et al., 1975). In some cases of severe immunosuppression e.g., in renal transplant patients, or individuals with genetic T-cell defects, anti-EBNA may be undetectable even though VCA-specific IgG antibodies are maintained at moderate or even high titres (Henle et al., 1973).

The expression and extent of expression of EBV infections as IM, BL or NPC is therefore very distinctly associated with age, socio-economic status, the immune status of the host as well as probable genetic predispositions to the diseases.

The EBV-susceptible cell(s)

The initial target cells of infection during IM remain to be identified. On the basis of in situ hybridization tests with exfoliated oropharyngeal epithelial cells from IM patients, Lemon et al., 1977 have claimed that these cells support replication of EBV. However, additional evidence is needed to substantiate this conclusion.

Among BL subjects, the initial cell for transformation is thought to be a B-lymphocyte. Klein et al., 1968 found that cells from the BL-derived LCL Daudi, were sIg(μ) and sIg(κ) positive, suggesting that sIg positive cells are indeed susceptible to EBV-induced transformation. Jondal et al., 1973 have shown that $sIg^{+}Fc^{+}$ B-cells but not T-cells bear EBV receptors among IM-, BL- and other EBV-transformed LCLs as well as cells from HPB. These data strongly point to the involvement of the B cell in EBV-induced oncogenesis.

The application of chromosome banding technique to BL- and non-BL-derived LCLs has revealed a constant structural abnormality of chromosome 14 (Manolov et al., 1972; Jarvis et al., 1974). This abnormality has been shown to be due to a translocation from No. 8 chromosome to give an extra band on both long arms of chromosome 14, designated t(8q-; 14q+)*. However, the No. 14 chromosome has been shown to be particularly unstable. A similar abnormality has been found among other types of malignant lymphoma e.g., lymphosarcoma, reticulum cell carcinoma, Hodgkin's lymphoma, and follicular lymphoma (Fukuhara et al., 1976) as well as after in vitro cultivation of lymphocytes which had been taken from malignant and non-malignant cases (Beatty-de Sana et al., 1975). It could therefore be that, B-cells carrying the spontaneous mutation t(8q-; 14q+) increase in number during the lymphoid stimulation of chronic malaria.
Infection of a cell of this kind by EBV in malarial cases, brings about the transformation event, leading to the production of a

*(Zech et al., 1976)

clone, expressing EBV-determined nuclear antigens.

The target cell for transformation among NPC subjects is not known, although infiltrating lymphocytes have been found in these patients and the EBV DNA has been detected in the epithelial cells of the tumour although they are devoid of EBV receptors (Wolf et al., 1973). The role of the infiltrating lymphocytes could be the involvement in the immune response against antigens which are specified by the virally-transformed cell.

Tonigaki et al., 1976 established LCLs from the peripheral blood leukocytes of myelogenous leukaemia. They found that some cells had the appearance of immature plasma cells and EM revealed that their structure was similar to that which was characteristic of plasma cells. No microscopic characteristics have been found by Moore et al., 1968 which seperate cultured human lymphoblastoid cells which had been derived from seropositive individuals and from patients with leukaemia, BL or IM.

Integration of EBV genome

Integration of the linear EBV DNA is thought to occur between 0 and 3 days after infection (Epstein et al., 1979). The mechanism for integration is not fully defined but a model has been proposed (Epstein et al., 1979). Modification of the linear EBV DNA is thought to occur prior to integration and there are specific host attachment sites on each DNA molecule which are available for integration. The substrate DNA for integration is covalently closed, double-stranded and supercoiled. Supercoiling may alter the secondary structure of attachment sites and thus render them accessible for the enzymes catalyzing the integration event. Exonucleases are then involved to generate the sticky ends, which promote the circularization of the molecule (Kintner et al., 1979).

EBV Strains and EBV-associated Diseases

The resident EBV DNA in NPC tumour cells, BL-derived tumour cells and lymphoid cells which have been obtained from IM patients occur partly as free episomes and partly as integrated sequences (Kaschka-Dierch et al., 1976; Lindhal et al., 1976; Adams et al., 1975; Pagano et al., 1975). There is therefore no difference in principle, between the way in which the viral genome associates with the DNA of a BL, an NPC or a lymphoid cell which has been derived from an IM patient.

Recently, Bornkamm et al., 1980 analysed the EBV DNA fragments which had been isolated from BL-derived P_3HR-1 and CC34-5; IM-derived B958; NPC-derived M-ABA and myeloblastic leukaemia-derived QIMR-WIL LCLs, with restriction endonucleases. They found that :-

(1) all strains studied were closely related

(11) the number of internal repeat sequences was variable among and within viral strains

(111) EBV B958 was the only strain with a large deletion of about 10,000 base pairs at the right-hand side of the molecule. At the same site, small deletions of about 400-500 base pairs were observed in EBV P_3HR-1 and EBV M-ABA DNAs.

(1V) EBV P_3HR-1, the only non-transforming EBV strain, had a deletion of about 300-400 base pairs.

(V) small inserted sequences of 150-400 base pairs were observed in M-ABA and EBV (B958) DNAs.

(V1) the cleavage patterns of P_3HR-1 virus DNA and the results of blot hybridizations with P_3HR-1 virus fragments were not conclusive and pointed to the possibility that in addition, some viral sequences may be arranged differently.

Although it is possible therefore that small differences in the genome organization of EBV may have significant biologic effects, the great similarity among different EBV strains does not favour the hypothesis that disease-specific subtypes exist.

FUNCTIONAL CHANGES AND EBV-TRANSFORMED CELLS

Immunoglobulin Production

Whether or not EBV-transformed B-cells possess antibody activity against available common antigens is within dispute. Kamei et al., 1968 reported that cells of the BL-derived cell line P_3J, produced Ig proteins which suppressed plaque formation by bacteriophage T_2. On the other hand, Krueger et al., 1974 could not find any significant neutralizing activity which would suggest that P_3J had anti-T_2 activity. Steel et al., 1974 have presented evidence, though unconfirmed, which would suggest that the proliferation of EBV-transformed LCLs in vitro may be responsible for the production of agglutinins which are directed against fixed sheep red blood cells (SRBC).

Induction of specific antigens can be brought about under appropriate in vitro conditions with EBV-transformed LCLs. Luzzati et al., 1977 found that, by adding SRBC and EBV-infected HPBLs to an in vitro system, EBV-transformed cells were obtained with anti-SRBC antibody activity. Similarly, Steinitz et al., 1977 succeeded in establishing EBV-transformed cells which produced anti-hapten (anti-NNP) antibody from a population of surface anti-hapten antibody-carrying lymphocytes, which had been preselected from non-antibody expressing cells by rosetting with NNP-coated erythrocytes. It is therefore probable that most specific antibodies can be induced with EBV-transformed cells under specific environmental conditions.

Lymphokines

The supernatant fluid recovered after stimulation of sensitized lymphocytes with antigen possess activity some of which have been ascribed to different molecular species. Among them are:-

(1) macrophage migration inhibition factor (MIF) which discourages the migration of macrophages from the site of antigen-mediated lymphokine release

(11) lymphotoxin which has marked cytotoxic activity and is thought to be associated with inhibition of tumour growth

(111) inhibition(s) of lymphocyte blastogenesis

The production of lymphokines have been shown to be a functional characteristic of EBV-transformed LCLs (Papageorgiou et al., 1974; Granger et al., 1970). Granger et al., found that 26 of 39 EBV-transformed LCLs produced lymphotoxin. Han et al., 1975 found that, inhibitory effects of cell-free supernatants from EBV-transformed LCLs on blastogenesis was significantly higher than that of supernatants from T-lymphocytes or from non-neoplastic cells.

The B-cell might not be the only cell which is susceptible to EBV-induced transformation, since NPC-derived epithelial cells have been shown to habour the EBV-DNA. However, the evidence which has been presented to date has shown conclusively that the B-cell is the only cell type which bears the EBV receptor.

Additional Discussion

Using the experimental conditions which have been outlined in this thesis, transformation was recognized by a change in the morphology of the infected cells from small mononuclear cells to cells with blastoid morphology. When in suspension as single cells. EBV (B958) transformed cells were found to be larger than EBV (QIMR-WIL) transformed cells. EBV (QIMR-WIL) transformed cells on the other hand, could adhere to culture flasks while EBV (B958) transformed cells were non-adherent. Moore et al., 1968, found that among cultured EBV-transformed cells which had been derived from seropositive individuals or from patients with leukaemia, BL or IM, characteristic morphological differences could not be recognized. Katsuki et al., 1975 on the other hand found that HUCLs which had been transformed by two different strains of EBV, displayed different morphological characteristics in suspension. It is therefore highly conceivable that different strains of EBV can induce different morphological characteristics in infected cells, depending on the age of the donor - HUCLs being more susceptible to morphological variations than HPBLs.

Transformation in the system which was used for this thesis could also be recognized by the appearance of the EBV-induced antigen, EBNA. The appearance of EBNA was first observed on day 2 after infection. Other workers (Thorley-Lawson et al., 1978; Aya et al., 1974; Yata et al., 1975) have shown that EBNA is first detectable between 0 and 3 days after infection. Integration of the viral genome is thought to occur between 0 and 3 days after infection of EBV (Epstein et al., 1979). It is therefore probable that integration of the viral genome into the host's chromosome is a necessary prerequisite for EBNA expression and the time of expression of EBNA is dependent on the time of integration of the infecting viral genome. A consequence of integration of the viral genome is the direction of synthesis of EBNA. Continual synthesis of EBNA then maintain transformation at the translational level.

There is no evidence to date which would suggest that EBV-infected cells are induced to synthesize Ig, once transformation has been initiated. In fact, Steel et al., 1977 have purported that the Ig synthesizing cells among EBV-infected cells are the Ig synthesizing cells in vivo. The findings of Lazatti et al., 1977 and Steinitz et al., 1977 would suggest that in the presence

of appropriate antigens EBV-susceptible cells could be induced to synthesize Ig in response to these antigens. Among long-term cultures of EBV (B958) and EBV (QIMR-WIL) transformed cells, I found that, populations of EBV-transformed cells had higher percentages of Ig synthesizing cells when compared with those among short-term cultures. These populations of EBV-transformed cells were thought to be synthesizing Ig in response to EBV-induced antigenic stimulation, since populations of cells with high percentages of EA-positive cells paralleled populations with high percentages of Ig synthesizing cells.

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